

2016

Epicardial Cell Engraftment And Signaling Promote Cardiac Repair After Myocardial Infarction

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EPICARDIAL CELL ENGRAFTMENT AND SIGNALING PROMOTE CARDIAC REPAIR
AFTER MYOCARDIAL INFARCTION

A Dissertation Presented

by

Krithika S. Rao

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Cellular, Molecular and Biomedical Sciences

January, 2016

Defense Date: November 17th, 2015
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ABSTRACT

The epicardium is a single layer of epithelial (mesothelial) cells that covers the entire heart surface, but whose function in adult mammals is poorly understood. Defining the role of epicardial cells during homeostasis, growth and injury has potential to provide new treatment strategies for human diseases that result in heart failure, due to extensive loss of viable cardiac tissue. We hypothesized that epicardial cells contribute to repair as transplantable progenitor cells for cellular regeneration and as a source of secreted growth factors for cell protection after myocardial infarction.

Adult epicardial cells were prospectively isolated as uncommitted epithelial cells using epithelial-specific beta-4 integrin (CD104). These cells underwent epithelial to mesenchymal transformation in culture to generate epicardial cell derivatives (EPDCs). We demonstrate that the C-terminal peptide from Connective Tissue Growth Factor (CTGF-D4), when combined with insulin, effectively primes EPDCs for robust cardiac engraftment in rats and contributes to improvement in cardiac function at one month after MI. Furthermore, we define a signaling axis comprised of CTGF-D4, low density lipoprotein receptor-related protein 6 (LRP6), sex determining region Y-box 9 (Sox9) and Endothelin Receptor B (ETB_R) that controls several key processes that impact EPDC graft success: cell survival, proliferation and migration. Interestingly, conditional deletion of ETB_R using epicardial-specific transgenic mice prevented epicardial cell proliferation and migration into myocardium after MI. We therefore observed a congruence in the signals and signaling pathways that control the proliferation and migration of endogenous EPDCs after MI and EPDCs that can be generated in cell culture and grafted back to the heart.

To gain additional insight into the cellular contribution of the epicardium, we utilized a non-injurious running exercise model to evaluate epicardial activity as a consequence of cardiac hypertrophy (i.e. myocardial growth model). We employed an inducible lineage-tracing system to specifically label and track epicardial cells by GFP expression. Prolonged exercise resulted in a significant number of GFP-positive proliferating epicardial cells and epicardial-derived GFP-positive endothelial cells and few GFP-positive smooth muscle cells in the heart. These observations highlight the cellular plasticity of the adult epicardium and its function as a cardiac progenitor cell niche, maintaining a source of replacement cells.

To investigate the paracrine properties of adult epicardial cells for their role in cell protection after MI and reperfusion, human epicardial cells were isolated from donor atrial tissue explants. We predicted that medium conditioned by cultured epicardial cells (EPI CdM) contained secreted reparative factors that would promote endothelial cell survival. Administration of EPI CdM promoted endothelial cell survival in culture and *in vivo*, 24 hours after ischemia-reperfusion injury. By screening EPI CdM, we detected protein complexes containing hepatocyte growth factor (HGF) with polyclonal IgG that imparted vascular protection *in vivo* in a manner similar to EPI CdM.

Overall, the studies presented here illustrate the unique biology of epicardial cells, their signaling networks, and their contribution to cardiac cell protection and regeneration. Importantly, these properties have the potential to be exploited in translational applications for cardiac repair.

CITATIONS

Material from this dissertation has been accepted for publication in the following form:

Rao KS, Aronshtam A, McElory-Yaggy KL, Bakondi B, VanBuren P, Sobel BE, Spees JL. (2015) Human epicardial cell-conditioned medium contains HGF/IgG complexes that phosphorylate RYK and protect against vascular injury. *Cardiovascular Research*. 107(2):277-86.

Miao T, Rao KS, Spees JL, Oldinski RA. (2014) Osteogenic differentiation of human mesenchymal stem cells through alginate-graft-poly (ethylene glycol) microsphere-mediated intracellular growth factor delivery. *Journal of Controlled Release*. 192:57-66.

Iso Y, Rao KS, Poole CN, Zaman AK, Curril I, Sobel BE, Kajstura J, Anversa P, Spees JL. (2014) Priming with ligands secreted by human stromal progenitor cells promotes grafts of cardiac stem/progenitor cells after myocardial infarction. *Stem Cells*. 32(3):674-83.

Material from this dissertation has been submitted for publication in the following form:

Rao KS, Kloppenburg JE, Marquis TD, Spees JL. (2015) CTGF-D4 engages an Lrp6/Sox9/ETBR signaling axis that promotes the epicardial response to injury and graft success after myocardial infarction. November 2015

ACKNOWLEDGEMENTS

Scientific pursuit in search of the truth, combined with the goal of securing an advanced academic degree is an enormous challenge. Fortunately for me, numerous people have significantly simplified and enriched this path over the years in their own unique ways.

I sincerely thank my advisor, Dr. Jeffrey Spees for being an extremely inspiring mentor and teacher. Jeff, I admire your passion, enthusiasm and remarkable creativity. Thank you for supporting my learning, research and career. You have motivated me to constantly think about great science and perform experiments and write effectively. Your scientific philosophy will always remind me to seek more knowledge and resources, with the ultimate goal of serving mankind. I will miss our constant exchange of ideas, shared excitement for new data and your characteristic humor.

I am grateful to my dissertation thesis committee members Dr. Alan Howe, Dr. Robert Kelm Jr. and Dr. Mercedes Rincon for their involvement in my performance in graduate school. I thank you all for your time and commitment towards improving the quality of my work and being great scientific and academic role models.

I am thankful for a set of wonderful labmates over the years, Matt LeComte, Sasha Aronshtam, Issei Shimada, Ingrid Curril, Jessica Kloppenburg, Taylor Marquis and Andrea Bibeau, for their advice, ideas, data collection, criticism, and writing. Your coffees, company and conversations made work feel like home. Special mention of Keara McElroy-Yaggy for contributing her fantastic surgical and animal husbandry skills in completing my projects. Keara, our relation evolved from being coworkers to family, and I thank you for your efforts, teaching, laughter, friendship, and moral support.

I appreciate the guidance and influence of other outstanding faculty at UVM including Dr. Tom Jetton, Dr. Mina Peshavaria, Dr. Nick Heintz, Dr. Matthew Poynter, Dr. Albert van der Vleit, Dr. David Schneider and Dr. Burton Sobel who motivated me to strive for excellence. I thank Dr. Ruth, Jennie, Roxana, Heidi, Erin, Kirstin and Becky for ensuring critical technical and administrative resource support, and importantly, their warm smiles and kind words. I fondly remember and acknowledge the roles that my high school and college teachers Dr. Shoba, Dr. Maha, Dr. Chitra, Dr. Julia, Dr. Murali and Dr. Hema played in nurturing my passion for science.

I am extremely appreciative of my invaluable personal friendships with Shruthi, Elianne, Ash, Saurav, Jean Luc, Matt Randall, Divya (x2), Ramya, Rajiv, Anbu, Arunima, Lyndelle, Niki, Madhav, Kamlesh, Subhodeep and Shadan. Each of you provided unrivaled support by filling these years with memories and flavors of essential emotions.

A special thanks to my local family in New Jersey for their support and care, without whom my survival would have been impossible. A special mention of my brother Suparth who has been a silent motivating force and the biggest keeper of good humor and all-things-good. My parents, whom I hold in the highest regard of relations, are a tremendous source of inspiration and support. From you I have learnt patience, hard work, confidence, and grace among many other things that have served me well at work. I am grateful to you, Bapa and Amma for all the opportunities you created, and experiences you enabled towards this goal. Thank you dear Raghu, for your knowledge and undying love, companionship and support. I particularly value your presence in seeing me through the critical last leg of this journey (and the timely gift of a replacement computer for my thesis writing!).

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LIST OF ABBREVIATIONS

MI	Myocardial Infarction
CHF	Congestive Heart Failure
LV	Left Ventricle
EF	Ejection Fraction
CSC	Cardiac Stem Cell
CPC	Cardiac Progenitor Cell
MSC	Mesenchymal Stromal Cell
CTGF	Connective Tissue Growth Factor
HGF	Hepatocyte Growth Factor
EPDC	Epicardial Derived Cell
LRP6	Low density lipoprotein Receptor-related Protein 6
ETB _R	Endothelin Receptor B
RYK	Receptor-like Tyrosine Kinase
EMT	Epithelial to Mesenchymal Transformation
SFM	Serum Free Medium
TM	Tamoxifen
CdM	Conditioned Medium
FGF	Fibroblast Growth Factor
IgG	Immunoglobulin G
ELISA	Enzyme Linked Immunosorbent Assay
kDa	kilo Dalton
PBS	Phosphate-buffered Saline
MI/R	Myocardial Ischemia/Reperfusion
LAD	Left Anterior Descending Artery
CD	Cluster of Differentiation
K18	Keratin 18
cKO	conditional Knockout
GFP	Green Fluorescent Protein

CHAPTER I. INTRODUCTION

Clinical need for translational research

Cardiac ischemia or myocardial infarction in humans

Ischemic heart disease that includes myocardial infarction (MI) is a leading cause of death worldwide among both men and women. In 2012, there were an estimated 7.4 million deaths due to ischemic heart disease, three quarter of which occurred in low and middle income countries (<http://www.healthdata.org/gbd>). In the United States there are an estimated 735,000 people that have an MI every year, with 120,000 cases resulting in death. Despite an about 40% fall in mortality rates due to MI over the last decade, it remains the leading cause of morbidity and healthcare burden on the surviving population (AHA Report, 2015). The risk factors for MI are prevalent at alarmingly high rates across multiple countries, thereby highlighting the paramount need for effective prevention and treatments.

Myocardial ischemia occurs during interruption of blood flow to the myocardium (heart muscle, cardiomyocyte), creating a mismatch between the myocardial oxygen demand and supply in the tissue. Ischemia is often caused by atherosclerotic plaque formation in the large coronary arteries. Plaque formation is a slow and chronic process responsible for narrowing of the coronary blood vessels and consequent decrease in blood flow through them. Myocardial infarction (MI) refers to the necrosis (death) of the cardiomyocytes as result of a sudden and profound ischemia (www.mayoclinic.org). An MI results when there is a sudden disruption of an atherosclerotic plaque that results in

lodging of plaque material (thrombotic occlusion) in the coronary artery. This occlusion causes severe ischemia and subsequent necrosis (infarction) of the myocardium.

Ventricular remodeling and cardiomyopathy

An MI creates a central zone of necrotic non-viable myocardium with a surrounding peri-infarct region consisting of viable, but ischemic tissue. The peri-infarct region represents myocardium that can be salvaged by timely restoration of blood supply through the occluded vessels (reperfusion). A greater time delay between an infarction event and reperfusion results in a corresponding increase in the expansion of the area of necrosis into the bordering ischemic but viable peri-infarct regions (Lamas, 1993; Litwin et al., 1994; McCormick et al., 1994; Nakamura and Tohma, 1991). A single event of MI can result in the death of around a billion cardiomyocytes (Sabbah et al., 1998). Widespread cardiomyocyte cell death causes the left ventricle (LV) wall to thin, thereby increasing the pressure and volume load on the heart. To compensate for this structural change, the LV wall undergoes dilatation that originates from the infarct region and spreads through the chamber.

The formerly healthy, contractile cardiomyocytes are replaced by noncontractile, akinetic fibrotic scar tissue comprised of myofibroblasts, which are beneficial to maintain the pumping efficiency of the heart and cardiac output in the early phase after MI (Bryant et al., 2009; Freed et al., 2005; Vasquez et al., 2010). However, over time increasing stress on the surviving cardiomyocytes and extensive scarring results in compensatory changes in shape, size, mass, volume and physiology of the LV. This is referred to as “negative” LV remodeling because it adversely affects cardiac function. Though

cardiomyocyte necrosis is the major cause of negative remodeling, death of blood vessels, fibroblasts and other cell types also contribute to subsequent changes in physiology (Roberts et al., 1984). Treating the natural progression of this disease remains a major clinical challenge and demands development of new therapies to preserve or replace of viable tissue.

Clinical diagnosis of MI

MI can be categorized on a clinical basis or on an anatomical and histological basis. In the clinic, MI is classified based on the electrical activity in the heart from electrocardiographic (ECG/EKG) measurements. These clinical classifications provide guidelines for the course of treatment and patient management. Anatomically, MI can be either transmural or non-transmural. A transmural infarct spreads across the 3 main heart layers in a cross section: the outermost epicardium, the middle myocardium (thickest layer) and the inner endocardium. A non-transmural MI is limited to the endocardium and inner myocardium, which are also morphologically the least vascularized regions in the heart (Figure 2) (www.mayoclinic.org). Echocardiography (ECHO) is a commonly used diagnostic tool for visualizing cardiac function using two- or three-dimensional and Doppler ultrasound visualizations. Of note, in addition to its clinical usage, ECHO is a commonly used method for evaluating the efficacy of basic and translational research discoveries.

Current treatment for MI

The current standard of care is determined on a patient-to-patient basis with the common goals of: (a) rescuing as much of the jeopardized myocardial tissue as possible, (b) relief

from angina (pain) and (c) prevention of further injury or related complications.

Reperfusion therapy for the prompt restoration of blood flow and oxygenation to provide relief from symptoms is performed in patients by mechanical or chemical interventions.

Percutaneous coronary intervention (PCI) is a commonly performed non-surgical procedure for the re-canalization of macroscopic coronary arteries. In certain severe cases, Coronary Artery Bypass Grafts (CABG) are performed to restore cardiac flow by creating new routes of blood flow through vascular grafting (Lamas et al., 1995; Pfeffer et al., 1992; Rutherford et al., 1994). Patients are also administered thrombolytic drugs which chemically dissolve a clot to release the occlusion and prevent further thrombosis (e.g. aspirin, IV unfractionated heparin, tissue Plasminogen Activator, IV streptokinase) and drugs for symptom relief and prevention of further infarctions (e.g. nitroglycerides, beta-blockers, ACE inhibitors) (Lamas et al., 1995; Pfeffer et al., 1992; Rutherford et al., 1994). In the USA, these mechanical and chemical interventions combined with improved emergency response and primary care have thus far achieved over a 95% survival rate for any given individual arriving to the hospital after an MI. Still, however, 1 out of every 5 survivors develops chronic heart failure (CHF) in the long-term and faces a 5-year mortality rate of around 50% (AHA, 2015 Report).

Heart Failure

Congestive heart failure (ischemic cardiomyopathy) results from necrosis of myocardial tissue after recurrent bouts of myocardial ischemia with infarctions. The current clinical treatment for CHF is limited to administration of pharmacologics that manage disease progression and extend patient life span (Atkinson and Robertson, 1979). Heart

transplantation is a strategy to replace a damaged organ with newer and more viable one, however this option is available to less than 0.1% of heart failure patients. Treatment of CHF to arrest the progression of heart disease or to completely eliminate it is therefore of critical importance. Cardiovascular disease has become a global epidemiologic issue due to the extent of the affected population, its life-long impact, and its social and financial demands for patient management.

Cardiac cell replacement by transplantation and engraftment of reparative cells

Early work on myocyte cell engraftment for MI

Over the last 2 decades, strategies to replace damaged cells in the heart with newer ones to prevent or slow the progression of heart failure after MI have received much attention and investment. The successful transplantation of skeletal myoblasts into damaged muscle of rats, mice and pigs inspired the possibility of providing healthy myocytes after MI, by direct cell injections into the heart (Schwarz et al., 1998). Experimental subjects were immunosuppressed for allogenic transplantation in different animal models. Direct delivery of myocytes or skeletal myoblasts was performed in rabbit and pig models of MI (Atkins et al., 1999; Azarnoush et al., 2005), as was delivery of postnatal or fetal myocytes (to harness their superior proliferative ability), into rats with MI (Azarnoush et al., 2005; Reffelmann et al., 2003). In theory, the best strategy would provide a cell source that could: (a) stabilize the existing tissue and prevent remodeling, (b) provide regional or global improvement in cardiac function by supporting myocyte contractility, and/ or (c) serve as a carrier for drugs or growth factors for molecular support to the host myocardium (Schwarz et al., 1998). However, transplantation of myocytes required their

survival and integration with the existing network of electrically coupled myocytes and the formation of intracellular connections gap junctions. In certain instances, engraftment of mature myocytes resulted in subsequent arrhythmias due to failure of the grafted cells to couple to the existing myocardial network. Altogether, outcomes from human clinical trials using muscle cells and fetal-derived cells did not generate much enthusiasm, but did open avenues for new treatment strategies (Hagege et al., 2006; Ozbaran et al., 2004; Patel et al., 2005). Identifying alternate cell sources for repair became an immediate need for the advancing field of cardiac cell therapy.

Stem cells as candidates for cell therapy

The myoblast transplantation trials coincided with developments in the field of stem cell biology, an area that was being explored for its massive potential for regenerative medicine. In general, stem/progenitor cells have properties of self-renewal, proliferation, migration and differentiation, which make them favorable candidates for cell therapy. For instance, using a well-defined cell surface marker profile (Visser et al., 1984), the hematopoietic stem cell (HSC) from the bone marrow could be isolated and expanded clonally *ex vivo* and *in vivo* (Dexter et al., 1977; Morrison and Weissman, 1994). By the 1970 and 80s, clinical allogenic and autologous bone marrow transplants with HSC were being carried out after complete irradiation. The HSC could self-renew (divide to replace itself while maintaining its differentiation potential), give rise to a population of fate-committed progenitors, and repopulate all lineages of the hematopoietic system due to its property of multipotency (Weissman and Shizuru, 2008). Accordingly, the administration

of naïve, undifferentiated stem/progenitor cells from bone marrow with benefit of differentiation into desired cell types gained interest for cell therapy of other organs.

Stem cell therapy for MI

Bone marrow-derived cells, circulating progenitors and MSCs for cardiac repair

In cardiology, numerous clinical trials were initiated using multiple candidate stem/progenitor cell types for engraftment. Cells were delivered to subjects either intravenously, through injections into the main coronary arteries, or by direct injection into the myocardium. Some moderate success was achieved after injection of bone marrow derived mononuclear cells. Success of these clinical trials was measured by improved cardiac function of patients early after cell administration (Klein et al., 2007; Pompilio et al., 2004; Stamm et al., 2003). Unfractionated bone marrow mononuclear cells were used due to ease of isolation, in addition to their capability of differentiating into vascular endothelial cells to restore perfusion and rebuild areas damaged by infarction. Improved methods to isolate, expand and culture other bone marrow-derived subpopulations such as vascular progenitor cells also initiated other clinical trials for cardiac regeneration in the heart. Another population, namely, Mesenchymal Stromal Cells (MSC) from the non-hematopoietic compartment gained widespread popularity in cardiac cell transplantation, in addition to their use in other organs (Amado et al., 2005; Chen et al., 2004; Giordano et al., 2007; Hare et al., 2009; Luan et al., 2010; Strioga et al., 2012). MSCs were particularly interesting because they could be propagated *ex vivo* to provide a source of multiple beneficial reparative secreted factors that were identified from their cell culture media (Boyle et al., 2010; Pittenger and Martin, 2004).

ES cells and iPSC cells for MI

Embryonic Stem cells (ES cells) which were first isolated in 1998 from the inner cell mass of the blastocyst during embryogenesis (Thomson et al., 1998). These cells retain the ability to differentiate into any desired cell type when exposed to appropriate hormones and growth factors (Ladd et al., 1998). Early transplantation studies with ES cells into animal models of MI resulted in teratoma formation and immune complications (Nussbaum et al., 2007). Further understanding of human ES cell biology helped to establish protocols for deriving cardiomyocytes *ex vivo* and *in vivo*, and coupling them with the existing myocardial structures after transplantation. These advances resulted in reports of 20-40% improvement in cardiac function for animal models of MI (Caspi et al., 2007; Laflamme et al., 2005; Shiba et al., 2012; Swijnenburg et al., 2005). To date, pluripotent cells or their derivatives have not been approved for cardiac clinical trials in the USA. However, one trial has begun in France using ES cell derivatives that are Islet1⁺/CD15⁺ progenitors derived from committed ES cells (www.clinicaltrials.gov).

A landmark discovery in 2006 by researchers in Japan (that was awarded the Nobel prize within 7 years) described methods to reprogram differentiated skin fibroblast cells into Pluripotent Stem Cells (induced PSC) by expressing 4 transcription factors (c-Myc, Klf4, Oct-3/4, Sox2) (Takahashi and Yamanaka, 2006). Eventually, these cells could be efficiently differentiated into highly pure cardiomyocytes using chemicals, gene expression or unique culture conditions and provided a valuable new resource for cell replacement (Pasha et al., 2011; Yu et al., 2007). iPSCs derived from mouse, human (Ieda et al., 2010) and several other organisms are now available to treat animal models and for preclinical and clinical testing for MI. Engraftment studies with iPSC in animal

models showed cardiac function benefits in addition to preservation of existing structures and coupling with myocytes (Dai et al., 2011; Mauritz et al., 2011). These cells overcome many of the limitations of ES cells for treating adults, while at the same time have numerous other applications (Guha et al., 2013). They can be used to create cardiomyocyte lines from different donor-derived iPSCs for genetic studies, drug screening and for creating patient-specific cell banks. For review, see (Lalit et al., 2014). However, utilization of iPSCs for clinical applications demands a significantly deeper understanding of cellular properties including clonal capacity, signals that control their proliferation and differentiation and their immune modulatory effects, among others.

Challenges to stem cell therapy after MI

Our ability to evaluate stem cell contribution in terms of direct cell replacement and benefit for cardiac function is currently limited. This is due to the low number of cells that initially engraft and survive, and that can be detected in the few months or even weeks after MI. In fact, thus far, in terms of cell engraftment and survival, poor results have been obtained with all candidate cell-types. Important 'issues' affecting cell survival and engraftment include the death of transplanted cells by apoptosis or necrosis, cell escape to extra-cardiac regions (this is of greater risk in the case of systemic cell injections) or mechanical loss from areas bordering the infarct due to constant pumping action of the heart (Wu et al., 2011). Although multiple clinical trials have reported modest improvement in cardiac function in patients after different times of follow-up, clear understanding of whether such functional improvements result from cell engraftment is lacking (Refer Table 1). To correlate the effects of cell engraftment

directly with cardiac function requires robust levels of initial engraftment, survival, proliferation, migration and (or) differentiation of the cells, and in some cases, their long term persistence in the heart. A recent report from 2014 in macaques showed modest cell survival at 1 month after MI (Chong et al., 2014). However, this was not a significant improvement in the level of engraftment over that achieved in prior pre-clinical studies in 2005 in sheep (Menard et al., 2005). Low engraftment and survival remains a major challenge for cell replacement after MI, as infarct necrotic zones are saturated with cell death signals, immune infiltrates, cell debris, redox mediators and extracellular exudates. Therefore, new strategies are required to promote cell survival and engraftment after MI.

Strategies to improve engraftment of transplanted cells in hearts with MI

Amongst various criteria, method of delivery is critical to determining the success of engraftment. To improve the efficiency of cell delivery, survival and persistence, additional manipulations are currently being tested prior to cell engraftment (Wu et al., 2011). For instance, any potential candidate cell can be preconditioned or primed by heat shock (Suzuki et al., 2000) or exposure to hypoxia, often termed “ischemic preconditioning” prior to engraftment (Chacko et al., 2010; Niagara et al., 2007). Grafted cells might also be genetically modified to express of pro-survival factors such as Akt (Kofidis et al., 2004; Kutschka et al., 2006; Mangi et al., 2003), or conjugated to magnetic beads to increase early retention (Cheng et al., 2010). Bioengineering material to serve as carriers and scaffolds for cell delivery has gained massive momentum to support cell therapy for MI. Grafted cells are frequently prepared by microencapsulation into degradable microbeads prior to engraftment (Paul et al., 2009). Alternatively, cells

are arrested in scaffolds where they are physically coupled to artificial or natural substances that form matrices, prior to injections. These matrices can either be injected as gels or deposited as patches on the heart surface (Guo et al., 2011; Wang et al., 2010). Most of these methods have shown promise in animal models of MI, where investigators have detected beneficial differences in cell injections with cells combined with engineered formulations versus cells alone (Bel et al., 2010; Hu et al., 2008; Huang et al., 2010; Tao et al., 2011; Yu et al., 2010). Notably, however, such constructs do have potential to interfere with the natural disease environment and to alter tissue in ways that could impede the natural course of remodeling and repair. Furthermore, the sustainable and practical aspects of these formulations have to be formally characterized prior to their use in humans.

An effective approach to improve cell delivery methods would likely benefit from the identification of biological signals in the cell's natural environment (niche) that supports its native survival and functions. As elaborated in this dissertation, providing sufficient or excess amounts of these endogenous signals during the process of engraftment supports cell survival and can foster their natural course of action. These signals can be delivered in controlled amounts, for specific periods of time, and in a cell-specific manner. Consequently, this directed approach eliminates the need to modify candidate cells genetically or by other means that risk abnormal behavior (e.g., unexpected tumors arising from primary cells due to gene overexpression). Harnessing cell signals from the injury environment has great potential to ensure predictable, safe and consistent outputs from grafted cells engraftment based on fundamental biological

properties, and may eliminate the variability and risks associated with grafting with artificial methods and products.

The second chapter of this dissertation presents new methods for successful grafting of cells to the adult heart after MI. The methods involve screening of niche regulating factors from cultures of mesenchymal stromal cells, and the ability of these factors (together or in defined, purified combinations) to prime cardiac stem/progenitor cells to promote robust cell engraftment. Our results demonstrate dramatic improvements in graft success obtained with any cell type to date, particularly for initial engraftment, proliferation, migration and differentiation of cells long-term after MI. Of note, cell engraftment into the subepicardial regions (beneath the activated epicardium) bordering the infarct was found to confer a unique advantage to cell survival and proliferation after engraftment. The new engraftment procedures were initially demonstrated with a population of widely-used c-Kit⁺ cardiac stem/progenitor cells, which can be isolated and propagated in culture to perform *ex vivo* studies to study mechanisms of cell signaling (Davis et al., 2010; Messina et al., 2004; Mishra et al., 2011). A cell surface glycoprotein, c-Kit (a.k.a. CD117), was a marker first used to prospectively isolate bone marrow stem cells (Ikuta and Weissman, 1992; Morrison and Weissman, 1994). In 2003, this marker was also first described to isolate a multipotent adult cardiac stem cell with the ability to proliferate and differentiate into multiple cardiac cell types including cardiac myocytes (Beltrami et al., 2003). These cells gained popularity from observations of repair in animal models following *in vivo* cell transplantation (Beltrami et al., 2003; Cimini et al., 2007; Fazel et al., 2006; Limana et al., 2005) and were quick to find their way into clinical trials for MI patients (Bolli et al., 2011).

<u>Cell Type/Study</u>	<u>Cell source</u>	<u>Clinical considerations</u>
Embryonic Stem cells	Embryonic tissue	Pre-clinical stage, considerations with linking to bioengineered substrate
Induced Pluripotent Stem cells	reprogramming	Pre-clinical stage, safety considerations
Differentiated cardiomyocytes	heart	No strategy for clinical studies
Fibroblasts	heart	Pre-clinical, transdifferentiation efficiency considerations
Cardiosphere isolated with markers	Cardiac biopsy	Stage I and II clinical trial
Mesenchymal stromal cells	Bone marrow biopsies	Stage II clinical trial
Bone marrow progenitor cells	Bone marrow biopsies	Stage III clinical trial
c-kit⁺ CSC	Cardiac biopsies	Stage II clinical trial
Epicardial cells	Cardiac biopsies	Non clinical considerations

Table 1. Summary of clinical trials for cardiac cell transplantation

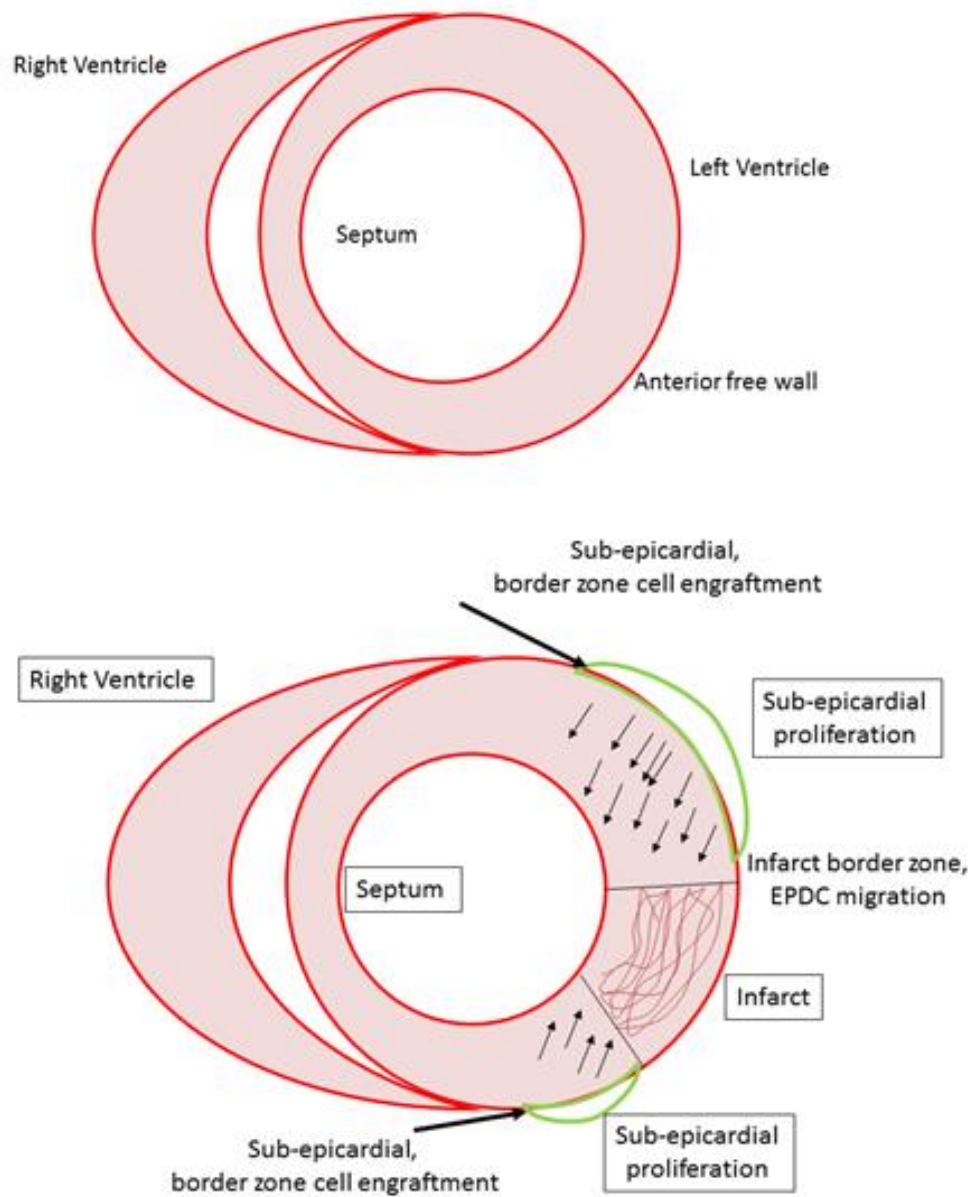


Figure 1- 1. Diagrammatic representation of the cross section of a mammalian heart indicating infraction and cell transplantation

Connective tissue growth factor in injury repair

CTGF is a 37 kDa protein first isolated in 1991 from cDNA expression libraries of human endothelial cells using anti-PDGF antibodies (Bradham et al., 1991). It has a widespread mRNA expression pattern as a single 2.4 kb transcript in heart, brain, kidney, placenta, liver and muscle (Surveyor and Brigstock, 1999) of adults. Within the heart, CTGF is produced mainly by myocytes and myofibroblasts (Ohnishi et al., 1998). Our group also detected transcripts for CTGF in purified epicardial cells. Although CTGF has been detected inside the cytosol of some cells, the majority of CTGF is secreted into the extracellular microenvironment (Brigstock, 2010). It can also be detected in plasma, serum and urine. The half-life of CTGF is not known, however its uptake was shown to be mediated by low-density lipoprotein receptor (LRP) (Brigstock, 2010).

CTGF is an unconventional growth factor, with a structure comprised of 4 distinct domains: an insulin-like growth factor binding protein (IGFBP) domain (module I), a Von Willebrand factor domain (module II), a thrombospondin-homology domain (module III), and a cysteine knot, heparin-binding domain (module IV). Each of these domains interact with multiple different extracellular matrix proteins and growth factors outside the cell, as well as integrins and receptors on the cell surface (Abreu et al., 2002; Grotendorst and Duncan, 2005; Tong and Brigstock, 2006; Yoshida and Munakata, 2007). Based on its numerous interaction partners, it is important to consider the activity of CTGF in a context-dependent manner (Brigstock, 2010). Angiopoietin II and Endothelin-1 induce CTGF expression during cardiac repair and potentially also function as downstream mediators of CTGF functions in angiogenesis (Leask and Abraham,

2006). Among its multi-faceted roles, there is growing evidence that CTGF promotes the process of epithelial to mesenchymal transformation (EMT) and coordinates receptor affinity and feedback signaling for BMP, and TGF-beta signaling and phosphorylation of SMADs (Leask, 2010). CTGF has been widely studied in the process of tissue fibrosis. While CTGF not capable of initiating fibrotic responses on its own, based on its interaction with TGF-beta it may function as a fibrotic “signal amplifier” (Accornero et al., 2015; Leask, 2015).

Cardiac Stem Cells

Does the heart have a bona fide stem cell? Questions and Controversies

The choice of cell to be grafted is a major consideration for success in cell therapy. The heart requires myocytes for contractile and electrical conduction, adventitial and interstitial fibroblasts for structural integrity, and vascular smooth muscle and endothelial cells to maintain perfusion. Engraftment of each of these cell types individually present unique issues, and engraftment of all differentiated cell types is complex and not feasible to perform clinically. As such, stem/progenitor cell sources that can provide multiple desired fates are more attractive for engraftment to the heart. While we are currently not limited by the available options for candidate stem/progenitor cell types for engraftment, advances in cell therapy with adult cells suggest that tissue-specific cell types will have the most benefit. Several stem/progenitor cell populations have been described in the heart that express c-Kit, Sca-1 (Oh et al., 2003), ATP binding transporter (Pfister et al., 2008) and that form cardiospheres that putatively mark a stem cell clone (Masuda et al.,

2012; Smith et al., 2007). However, none of these markers or properties are unique to the heart, nor are the methods such as cardiospheres isolation a reliable or specific way to obtain stem cells.

More recently, many have challenged the validity of c-Kit as a marker of a true stem or progenitor cell in the adult heart (Servick, 2014). Early reports demonstrating their ability to differentiate into cardiac myocytes, albeit in low numbers, helped them to rapidly enter into human to clinical trials (Beltrami et al., 2003). However, many investigators eventually failed to detect significant proliferation of c-Kit cells in the heart after MI. Combined with the absence of detectable myocytes that derived from c-Kit⁺ cells, further studies contradicted what was previously reported (van Berlo et al., 2014). To date, as the field has still been unable to reach a consensus regarding a true adult cardiac stem cell marker, and the opportunity for discovery remains.

Epithelial stem cells

A unique stem cell has not been identified for several adult tissues or organs in mammals. In fact, emerging reports are now challenging the existing dogma that proposes a true stem cell (by definition) exists in every organ. Instead, evidence from multiple organs indicates that a differentiated cell within some organs has the ability to dedifferentiate into a more primitive stem or progenitor-like phenotype and function in organ repair after injury (Blanpain and Fuchs, 2014). For instance Clevers *et. al.* identified a cell surface marker “Troy” that marks a specific subset of chief cells in the intestine that display plasticity (i.e. they serve as quiescent reserve stem cells and can replenish the entire gastric unit at the tissue level upon dedifferentiation) (Stange et al., 2013). Studies in the

lung demonstrated the ability of a committed epithelial cell to dedifferentiated and function as a stem cell *in vivo* after injury (Tata et al., 2013), while differentiated kidney epithelial cells were shown to participate in kidney repair after proximal tubule injury (Kusaba et al., 2014). These observations challenge the notion that stem cell hierarchies are represented by a unidirectional model for differentiation, and that committed cells can, in part, serve to be a part of the homeostatic and eventual tissue regeneration programs in the body.

Interestingly, the above examples also allude to the theme of epithelial cells serving as putative stem cells in multiple organs after injury reviewed by Blanpain and Fuchs (Blanpain and Fuchs, 2014). This review details the remarkable plasticity of epithelial cells that were thought to be lineage-restricted and their ability to acquire stem like properties during physiological or regenerative conditions. In the heart, among various differentiated cell types, the epicardium, an epithelial layer covering the entire heart surface has marked potential for dedifferentiation. Epicardial cells are promising candidates for engraftment based on myriad properties that include their role as a progenitor cell source in development, their direction of tissue morphogenesis by protein/peptide secretions, and emerging evidence for functions in modulation of inflammation after an injury (Smart and Riley, 2012).

Epicardial cell biology in development and disease

Epicardium forms the cover of the heart

The epicardium is a single layer of epithelial (mesothelial cells) that covers the entire heart surface. It plays key roles in signaling to the myocardium and also as a progenitor

cell source for different cardiac lineages. Physical removal of the epicardial layer in developing avian hearts resulted in arrest of cardiomyocyte growth, leading to a thin-walled ventricle, and the arrest of coronary vessel development (Gittenberger-de Groot et al., 2000; Pennisi et al., 2003). Likewise, knockout of genes required for epicardial survival and function in mice [e.g., *beta-catenin* (Zamora et al., 2007), *Wt1* (Guadix et al., 2011), *Vcam1* (*alpha4beta1 integrin*) (Sengbusch et al., 2002), *Gata4* (Watt et al., 2004), *Notch* (del Monte et al., 2011; Loomes et al., 2002)] resulted in similar phenotypes.

Epicardium during development

During cardiac development, the epicardium serves as an important signaling center to ensure myocyte development and a cellular source for formation of the coronary vasculature. Lineage-tracing studies in developing chick and quail embryos have provided insights into the origin and fate of the epicardium. It derives from the proepicardial organ (epithelium attached to the embryonic diaphragm), and in mice, around embryonic day 9.5 (E9.5) it migrates to completely engulf the myocardial and endocardial layers. Around E10.5 it forms an epithelial covering around the entire developing structure, termed the epicardium. From this stage onwards it is responsible for directing events that help to complete cardiac development.

Between E11.5 and 13.5, epicardial cells undergo epithelial to mesenchymal transformation, providing a population of subepicardial mesenchymal cells and precursors that migrate into the myocardium. Retinoic acid (RA) and erythropoietin (Epo) signaling within the epicardial cells stimulates them to secrete mitogens that are required for myocyte proliferation and maturation; this induces maximal heart growth.

Mice deficient in RA or Epo signaling were reported to have severe myocyte hypoplasia. In turn, it has been shown that epicardial PDGF, FGF9, FGF16 and FGF20 signaling to myocyte FGFR1 and 2 receptors mediates myocardial growth (Lavine and Ornitz, 2008; Lavine et al., 2005; Vega-Hernandez et al., 2011).

Epicardium in lower vertebrates

In lower vertebrates, which share conserved features of cardiac development with mammals, the role of the epicardium has been extensively studied. These organisms provide excellent model systems for genetic manipulations and real-time visualization of *in vivo* processes. The zebrafish heart is capable of completely regenerating damaged cardiac tissue (similar to many other organs), to provide a functionally perfect structure, identical to the former one. Resection of a piece of zebrafish ventricle leads to production of a blastema structure; this is then followed by activation of the localized epicardium (by re-expression of embryonic transcription factors). The activated cells then make a new epicardial covering around the blastema and then undergo EMT to provide progenitors for formation of vasculature in the repair area. FGF17 signaling from the disrupted myocytes through FGF receptors on the epicardium coordinated the repair process (Lepilina et al., 2006). More recently the same group of investigators used a similar transgenic zebrafish model that expressed bacterial nitroreductase (catalyzes conversion of a non-toxic substrate to cytotoxic product) in a cell-specific manner. This was used to ablate (deplete) the epicardial layer after myocyte injury (Wang et al., 2015). Compared with the course of repair in normal animals, in the transgenic animals, cardiomyocyte proliferation was inhibited, and long term regeneration of the cells was delayed.

Epicardium in mammals

During adulthood, the mammalian epicardial layer is thought to remain quiescent.

Although the role of the epicardium after injury to the heart has garnered much interest, our understanding of its function is quite poor. Thus far, after injury it is clear that a population of epicardial cell become activated and gain the expression of embryonic epicardial markers such as *Wt-1*, *Raldh2*, *Tbx18* and *Gata4*, factors not expressed in the normal adult heart. Lineage-tracing studies using these activated transcription factors as markers demonstrates migration of a subset of these cells into the myocardium. Winter *et al.* (2007) defined “spindle-like cells” obtained by removing the epicardial layer from human atrial tissue explants as “epicardial-derived cells” (EPDC) (Lie-Venema *et al.*, 2007; Winter and Gittenberger-de Groot, 2007). These cells were cultured and transplanted into the hearts of immunodeficient mice using a permanent ligation model of MI. They observed differences in various parameters of cardiac function up to 6 weeks after MI, however, they failed to detect engrafted cells at that time point (Winter *et al.*, 2007). Several studies in mice have demonstrated that after experimental MI, epicardial cells become activated to proliferate, undergo EMT, migrate into the myocardium and secrete factors that promote angiogenesis and fibrosis within and adjacent to areas with infarction (Gittenberger-de Groot *et al.*, 2010; Masters and Riley, 2014; Smart *et al.*, 2013; Zhou *et al.*, 2011).

Utilizing epicardial cell biology for cardiac cell transplantation

Based on our current understanding of organ resident adult stem cells and the known roles of the epicardium, this cell population satisfies certain key criteria to be defined as

stem/progenitor cells for the heart. Therefore epicardial cells and their derivatives can potentially be a source of cell replacement cells after MI or of mitogens that preserve and/or repair tissue after injury. To date, few studies have explored the potential of epicardial cells and their derivatives as candidates for cell engraftment. This is due, in part to, limitations in knowledge and lack of appropriate tools for epicardial cell-specific isolation and *in vivo* experimentation. In order to reproducibly isolate a pure population of cells from donors, epicardial cell-specific surface markers or epitopes need to be identified. These markers have to be unique to this population in the heart, similar to methods that identified progenitor cells in other organs (Prockop et al., 2003; Wang et al., 2015). Additionally, we need to investigate key changes in epicardial cells including signals received and emitted by the epicardial cells in an injury environment. Currently, understanding of intracellular and extracellular epicardial signaling has been limited to embryonic studies. However, there is need for a comprehensive examination of signaling events and pathways that control epicardial cell proliferation, EMT and migration in the adult, which can be eventually targeted to improve or modify injury repair. The third chapter of this dissertation focuses on answering key questions in adult epicardial cell biology that may help to utilize epicardial cells for effective cell replacement therapies. In order to assess the importance of epicardial cells in repair, our approach in these studies was focused on enhancing benefit from epicardial cells by means of cell transplantation and engraftment. This focus helped us identify a novel molecular target and signaling axis that controlled epicardial cell migration. Overall, our studies present an exciting research opportunity to determine mechanisms that advance therapeutic use of epicardial cells and their derivatives.

Epicardial progenitor cells

The fate of epicardial-derived cells after migration into the myocardium is the subject of numerous debates that stem from conflicting observations in different experimental models (Lie-Venema et al., 2007; Rudat and Kispert, 2012). Various lineage-tracing studies demonstrate that the majority of epicardial cells differentiate into cardiac interstitial fibroblasts and vascular smooth muscle cells that line large cardiac arteries and arterioles (Gonzalez-Rosa et al., 2012; Mikawa and Gourdie, 1996; Rudat and Kispert, 2012; van Tuyn et al., 2007). The epicardium has not been directly shown to contribute endothelial cells to the heart, but lineage-tracing studies report a population of cells from the proepicardial organ that differentiated into endothelial cells (Perez-Pomares et al., 2002; Wada et al., 2003). This raises the possibility that a second progenitor exists in the proepicardium that migrates separately and is distinct from epicardial-derived progenitor cell, based on its ability generate endothelial cells. By contrast, other experiments performed with quail and chick chimeras during embryogenesis suggest that the epicardial mesothelium itself is a source of endothelial progenitor cells. In these studies, retroviral and fluorescent reporter labeling comparing the derivatives of the proepicardial and epicardial compartments suggests that a portion of the population of coronary endothelial cells derive from the epicardial cells, after they undergo EMT (Perez-Pomares et al., 2002). An additional issue concern whether epicardial-derived progenitor cells can differentiate into cardiac myocytes (Cai et al., 2008; Kruithof et al., 2006; Zhou et al., 2012; Zhou et al., 2008) (Figure 2).

The fourth chapter of this dissertation addresses the cellular requirements of the adult heart in a running exercise model of cardiac tissue growth. We employed a cell

growth or hypertrophic model because it creates demand for additional perfusion and capillary formation. By exercising epicardial-specific lineage-tracing mice over a prolonged time period, we were able to track labeled cells as they migrated through the myocardium and adopted different cell fates including endothelial cells.

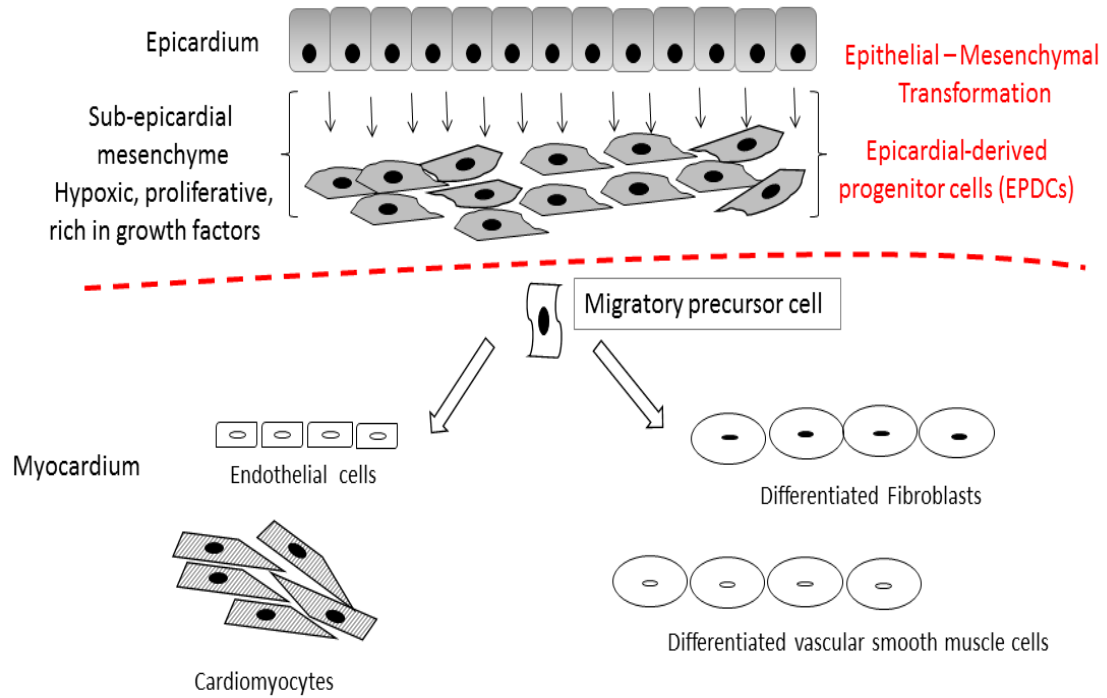


Figure 1- 2. Epicardial Epithelial to Mesenchymal Transformation

Epicardial cells undergo epithelial to mesenchymal transformation to generate a population EPDCs in the subepicardial mesenchyme. These cells proliferate here prior to their migration into the myocardium where they function as progenitor cells. They differentiate to generate adventitial fibroblasts and vascular smooth muscle cells, while their ability to differentiate into endothelial cells and cardiomyocytes needs to be definitively established

Vascular damage and protection as a target for paracrine biology

The phenomenon of “no-reflow”

In certain instances, reperfusion therapy after MI does not completely restore perfusion to regions of ischemia, especially to the inner myocardium lying closest to the endocardial layer (French et al., 2010; Zaman et al., 2011). Despite nearly complete restoration of blood flow through the major coronary arteries, areas downstream from the site of occlusion do not re-oxygenate. These areas of poor blood flow are referred to as regions of “no-reflow” or “low-reflow” and are also described during ischemia in other organs and tissues including brain, skeletal muscle, skin and kidney (Rezkalla and Kloner, 2002). The phenomenon of no reflow in the heart was first demonstrated experimentally in dogs in 1974 by performing proximal coronary artery ligation followed by reperfusion at 40 minutes or 90 minutes after ligation (Kloner et al., 1974). Whereas restoration of flow after 40 mins provided complete perfusion to the damaged areas, restoration of flow after 90 minute ischemia period resulted in only partial perfusion. Anatomical examination of these sites of “no-reflow” suggested significant microvascular cell death (i.e., vessels around 200 microns), breakdown of the endothelial barrier, and vascular leak. Such structural damage prevents localized blood circulation, causing edema and accumulation of toxic metabolites and immune infiltrates. Furthermore, dyes that were injected to record perfusion through myocardial tissue failed to trace into regions of no-reflow. This indicated definitive damage to the vasculature and uncoupling of the microvascular circulation from the systemic circulation.

No-reflow is associated with adverse clinical events and is now believed to affect later processes such as scar formation, ventricular remodeling and the development of future collateral flow (Rezkalla and Kloner, 2002). Although a complete understanding of the causes and effects of no-reflow on the heart is lacking, numerous theories have been put forth to explain the process. More recently, the TAPAS clinical trial has proven that prevention of angiographic microvascular damage during an MI may reduce overall cardiac injury and improve clinical outcomes (Vlaar et al., 2008). It has also been established that the extent of the area at risk of no-reflow directly correlates with increased times of ligation, and extent of initial tissue damage from ischemia. This has important clinical implications in that the damaged tissue that fails to receive blood will remain inaccessible to drugs or recovery by mechanical intervention (Tarikuz Zaman et al., 2013). Various interventions for reversing no-reflow following MI have been reported, including pharmacological drugs for vasodilation and mechanical devices for protection from embolisms. To date, however, no controlled randomized trials have been performed to determine the clinical benefit of these therapies. Therefore, there remains a great need to develop interventions that protect the structural and functional integrity of the microvasculature early after MI, which can potentially limit the extent of damage. One strategy gaining importance is the direct targeting of tissues with vaso-protective treatments to activate survival pathways in the endothelial cells. This approach has potential to salvage large areas of surrounding myocardial tissue at risk and to provide to long-term preservation of cardiac function (Tarikuz Zaman et al., 2013).

Paracrine effects of cell therapy

One important role for stem cells in tissues that has been extensively studied is their role as a source of paracrine factors. These cells secrete factors within their local environment to influence processes like tissue morphogenesis, preservation, and the activities of other stem or differentiated cells during tissue repair (Anthony and Shiels, 2013; Shimada and Spees, 2011). The role of paracrine factors in the injury microenvironment has been well-studied in the bone marrow. In adults, the bone marrow is the main center for hematopoiesis (i.e., formation of all blood cells), as it contains niches for the Hematopoietic Stem Cell (HSC). HSC survival, proliferation, differentiation and migration are controlled by niche factors in the bone marrow microenvironment. HSC niche factors are secreted primarily by the mesenchymal stromal cells (MSC) that reside in close physical proximity to the HSC (Anthony and Shiels, 2013; Ehninger and Trumpp, 2011; Gregory et al., 2005; Jones and Wagers, 2008; Phinney and Prockop, 2007; Prockop et al., 2010).

The niche factors produced by MSC can be harnessed for developing therapeutics because we can isolate, expand and cultivate them *ex vivo*. We then analyze and purify factors from their secretions and test them in large scale screens to identify drug candidates. These purified factors can be directly administered at optimal concentrations. This approach provides multiple advantages in terms of approval by FDA, controlled amounts for administration, and ease of storage and handling and administration. An important advantage of paracrine therapy over cell therapy is the ability to elicit acute treatment effects when compared to the long-term cell replacement or sustained effects of cell therapy, in addition to overcoming challenges of immune-mismatch (Gnecchi et al.,

2008). The paracrine role of stem cells is valuable for cell therapy applications, both to protect jeopardized tissue and to rebuild damaged structures.

Harnessing the paracrine biology of epicardial cells to treat MI

Beginning with the use of bone marrow-derived mononuclear cells, patients in many cell therapy trials for MI showed modest improvements in cardiac function including increased ejection fraction, fractional shortening, stroke volume and cardiac output (Kang et al., 2004; Wollert et al., 2004). The initial excitement over these improvements motivated numerous debates concerning the safety of autologous transplantation, benefit of intracoronary versus systemic delivery, and the logistics of isolating and expanding large cell populations to derive optimal benefit from the interventional procedure that the treatment demanded (Da Silva and Hare, 2013; Karantalis and Hare, 2015; Suncion et al., 2012). Among these concerns, the question regarding mechanism of action for cardiac cell therapy or, specifically, the changes at the cellular and molecular levels that influenced function has been an expanding field of investigation (D'Souza et al., 2015; Dai et al., 2007; Gneocchi et al., 2008; Haider et al., 2008; Mirotsov et al., 2007). Since myocytes are the cellular functional units of the heart, the overt expectation was that cell therapy worked to improve cardiac function by differentiation of stem cells into cardiomyocytes, thereby increasing their numbers. However, most trials failed to detect significant engraftment of transplanted cells. Furthermore, stem cell transplantation did not stimulate the proliferation of myocytes at numbers adequate to improve cardiac function. It should be noted that transplantation of myocytes alone has been performed by many investigators, but presents a formidable challenge in terms of ensuring *in vivo*

survival, proliferation and integration of grafted cells within the existing network of myocytes and electric coupling to prevent fatal arrhythmia. In the absence of large numbers of transplanted cells to account for the observed functional benefits, the paracrine hypothesis became a more plausible and accepted mechanism for stem cell function (Avolio et al., 2015; D'Souza et al., 2015; Dai et al., 2007; Mazhari and Hare, 2007; Menasche, 2015; Quijada et al., 2015; Wollert and Drexler, 2005, 2006; Yannarelli et al., 2014). The paracrine study of epicardial cells has been largely inspired by research of developmental processes where available literature indicates numerous paracrine signals from the epicardium guide myocardial development (Bochmann et al., 2010; Bollini et al., 2014). Gene expression changes also suggest that the epicardium is a notable source of cytoprotective and angiogenic factors (Huang et al., 2012; Lui et al., 2014; Velecela et al., 2013; Zhou et al., 2011). From the standpoint of MI, early therapies to limit infarct area and expansion by treating the extent of no-reflow can be a suitable treatment strategy for early vascular protection and to control late tissue remodeling (Tarikuz Zaman et al., 2013).

The fifth chapter of this thesis focuses on treatment of low- or no-reflow after an MI. The chapter introduces methods to evaluate endothelial damage that occurs due to no-reflow early after ischemia/reperfusion injury, followed by treatments to minimize this damage. Screens of epicardial cells isolated from human donors, to identify secreted repair and angiogenic factors, are also presented. One screen identified a powerful growth factor and its biochemical interacting partner that enhance vascular protection early after MI/R injury, and furthermore identified a novel receptor required for their full effect on endothelial cells.

Statement of specific aims

The process of cardiac regeneration involves coordinated changes in multiple cell-types in the heart, and occurs during tissue growth, after injury and during disease or ageing. Among the various cells that have been studied, the role of epicardial cells in adults, especially during regeneration is poorly understood. The overall goal of my dissertation was to investigate novel functions of the adult epicardium and demonstrate the important contributions of epicardial cells in cardiac regeneration. In each chapter, I focused on studying a distinct aspect of epicardial cell signaling and biology in a specific physiological context (e.g., during repair after MI or hypertrophic growth). In order to address specific research questions, my primary experimental objectives in this dissertation included: i] understanding endogenous epicardial cell biology, ii] identification of epicardial markers and signals to promote their utility for cell replacement therapy after MI, and iii] screening epicardial cells for secreted factors for early treatment of MI, in a manner which can restore cardiac function.

Significance of the models and experimental approach

Work carried out in this dissertation integrates the use of multiple animal species for *in vivo* studies combined with work on primary cells isolated from several different mammalian species including human. This approach has been adopted to overcome the apparent technical limitations of each model. Rat models are larger in size, facilitating engraftment and treatment studies requiring coarse manipulations, whereas mouse models are better established for performing genetic manipulations and studies that evaluate

endogenous cells. Notably, a variety of transgenic mouse models allowed us to perform genetic manipulations, and to evaluate the role of specific, novel factors in epicardial cell biology. The different animal models of injury and physiology enabled us to model both the onset and progression of cardiac injury through various stages, and the effects of different treatments at different time points. Evidence from multiple model systems increases confidence that our findings provide fundamental insight into the cell biology of multiple mammalian species and highlights shared similarities in key cellular properties. In addition, results from our studies highlight the power of using animal models to advance the understanding of tissue injury in humans and the natural process of repair.

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CHAPTER II. PRIMING WITH LIGANDS SECRETED BY HUMAN STROMAL PROGENITOR CELLS PROMOTES GRAFTS OF CARDIAC STEM/PROGENITOR CELLS AFTER MYOCARDIAL INFARCTION

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Abstract

Transplantation of culture-expanded adult stem/progenitor cells often results in poor cellular engraftment, survival, and migration into sites of tissue injury. Mesenchymal cells including fibroblasts and stromal cells secrete factors that protect injured tissues, promote tissue repair, and support many types of stem/progenitor cells in culture. We hypothesized that secreted factors in conditioned medium (CdM) from adult bone marrow-derived multipotent stromal cells (MSCs) could be used to prime adult cardiac stem/progenitor cells (CSCs/CPCs) and improve graft success after myocardial infarction (MI). Incubation of adult rat CPCs in CdM from human MSCs isolated by plastic adherence or by magnetic sorting against CD271 (a.k.a., p75 low-affinity nerve growth factor receptor; p75MSCs) induced phosphorylation of STAT3 and Akt in CPCs, supporting their proliferation under normoxic conditions and survival under hypoxic conditions (1% oxygen). Priming CSCs with 30x p75MSC CdM for 30 min prior to transplantation into sub-epicardial tissue 1 day after MI markedly increased engraftment compared with vehicle priming. Screening CdM with neutralizing/blocking antibodies identified Connective Tissue Growth Factor (CTGF) and Insulin as key factors in p75MSC CdM that protected CPCs. Human CTGF peptide (CTGF-D4) and Insulin synergistically promoted CPC survival during hypoxia in culture. Similar to CdM priming, priming of CSCs with CTGF-D4 and Insulin for 30 min prior to transplantation promoted robust engraftment, survival and migration of CSC derivatives at 1 week and 1 month after MI. Our results indicate that short-term priming of human CSCs with CTGF-D4 and Insulin may improve graft success and cardiac regeneration in patients with MI.

Introduction

Poor graft success is a common problem after transplantation of cultured cells into injured tissues and occurs with transplants of adult stem/progenitor cells, embryonic stem (ES) cells, and ES cell derivatives [1-3]. Despite rapid progress in methods to identify, isolate and culture candidate cells for tissue repair, the inability to effectively graft culture-expanded cells to diseased or injured adult tissues remains a challenge for many anticipated forms of cell therapy. Cell grafts for solid, non-hematopoietic tissues and organs such as the heart are particularly inefficient, especially after ischemic injury. Upon transplantation, culture-expanded cells can exhibit low adhesion to host tissue, low survival, and/or low levels of migration [2,3]. Improving these stages of cell engraftment is critical because they typically precede differentiation and functional integration of transplanted cells into host tissue. Recent efforts to improve graft success have utilized genetic manipulation to over-express pro-survival factors such as Akt in transplanted cells or co-administer cells with accessory materials/scaffolds to support the graft [4,5]. Paracrine activity from mesenchymal cells such as fibroblasts and other stromal cells promotes tissue repair after injury [6,7] and also regulates, in part, stem cell niches [8]. In the bone marrow, endothelial cells and stromal derivatives from non-hematopoietic progenitor cells (multipotent stromal cells, MSCs) support hematopoietic stem cells (HSCs) by providing critical structural and regulatory components of the hematopoietic niche. The niche components include cellular substrate, e.g. extracellular matrix, as well as multiple growth factors, cytokines, and hormones that influence HSC self-renewal, proliferation, survival, and function [8-12]. Due to their supportive roles, feeder layers of

stromal cells (e.g. MSCs or fibroblasts) are commonly used to support the culture of HSCs, other types of adult stem/progenitor cells, and ES cells [12-15].

MSCs are typically isolated from total bone marrow mononuclear cells simply based on their adhesion to tissue culture plastic. To standardize isolation methods, several investigators have sorted human MSCs from bone marrow aspirates based on cell surface epitopes such as CD271 (p75 low-affinity nerve growth factor receptor, p75MSCs) or CD133 (Prominin-1, CD133MSCs) [16-18]. In some cases, sorting MSCs based on markers appears to enrich subpopulations of MSCs that differ in terms of paracrine activity. Of clinical interest, the different repertoires of secreted proteins/peptides may enhance particular therapeutic applications. For example, secreted factors from the CD133MSC subpopulation was shown to provide greater protection of cerebral tissue after stroke compared with those from the p75MSC subpopulation [18]. In transplantation studies, co-infusion of human HSCs and p75MSCs into immunodeficient mice provided a 10 to 23 fold improvement in multi-lineage engraftment of bone marrow compared with co-infusion of HSCs and typical (non-selected) human MSCs [19].

CD271⁺ cells characteristic of bone marrow p75MSCs are rapidly mobilized into the blood of patients with acute MI [20]. We hypothesized that marrow-derived CD271⁺ cells participate in cardiac repair/remodeling after MI, in part, through paracrine activity. We investigated the effects of stromal cell-derived ligands on cardiac stem/progenitor cells (CSCs/CPCs) and found that conditioned medium (CdM) from human p75MSCs, but not from CD133MSCs, supported the proliferation and survival of adult rat CSCs/CPCs. Furthermore, priming of CSCs in p75MSC CdM for 30 min prior to transplantation markedly improved CSC grafts after MI. By screening p75MSC CdM

for molecules that protected CPCs under hypoxic conditions, we identified two ligands with synergistic effects on CSC survival and developed a novel priming tool to enhance graft success.

Results

CdM from human stromal cells induces proliferation of rat CPCs

CdM was collected from human MSCs, p75MSCs, and dermal fibroblasts. CdM from each of the cell types supported CPC proliferation (Figure 1A and Supplemental Figure 1). We observed a concentration-dependent increase in CPC number when CPCs were incubated in 10x-concentrated CdM (10x CdM) from MSCs or p75MSCs (Supplemental Figure 2A). In contrast, when CPCs were incubated in CdM vehicle (serum-free α -MEM, SFM), their numbers gradually decreased (Figure 1A and B). To confirm DNA synthesis and active cell cycle status, we quantified incorporation of BrdU into CPCs 24 hrs after exposure to CdM. The percentage of BrdU-positive CPCs in CdM from MSCs, p75MSCs or fibroblasts was significantly greater than that for SFM-treated CPCs (Figure 1B).

Immunoblotting demonstrated that Ki67 was expressed in CPCs treated with CdM but not in CPCs treated with SFM (Figure 1B). In contrast to its effects on CPCs, CdM from MSCs or p75MSCs did not support the proliferation of cardiac fibroblasts (Figure 1C).

CdM from human MSCs activates STAT3 and Akt in CPCs. STAT3 activation is critical for self-renewal of ES cells, adult HSCs, and adult neural stem cells [22-24].

Accordingly, we assayed levels phosphorylated-STAT3 (p-STAT3; Tyr705) in CPCs exposed to CdMs from MSCs, p75MSCs, or fibroblasts. Levels of p-STAT3 were significantly higher in CPCs at 1 and 2 days after CdM treatment compared with SFM

(Figure 2A). Immunocytochemistry demonstrated that p-STAT3 (Tyr 705) localized to the nuclei of CPCs treated with CdM (Figure 2B). In addition to p-STAT3, we observed phosphorylation of Akt (p-Akt; Ser473) after incubation of CPCs in CdM from each of the stromal cell types (Figure 2C). However, we also observed p-Akt in CPCs incubated in SFM alone (Figure 2C). Notably, auto-phosphorylation of signaling molecules affecting cell survival such as Akt, ERK1/2, and mTOR occurs in diverse cell types during serum or nutrient deprivation [25].

To determine whether p-STAT3 and/or p-Akt mediated the effects of CdM on CPCs, we treated CPCs with pharmacological inhibitors. AG490, the Jak2/STAT3 pathway inhibitor, reduced the number of CPCs treated with MSC CdM in a dose-dependent manner: control (CdM+DMSO), $100 \pm 1.5\%$; $1 \mu\text{M}$, $96.3 \pm 0.9 \%$; $5 \mu\text{M}$, $89.5 \pm 0.9 \%$; $10 \mu\text{M}$, $43.4 \pm 2.4 \%$ (cell number ratio to control cell number [121,863 cells], mean \pm SEM, $n = 3$ to 6). The inhibitory effect of AG490 was also observed for CPCs treated with CdM from p75MSCs or fibroblasts (Figure 2D). LY294002, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway inhibitor, decreased CPC number in CdM but to a lesser extent than did AG490 (Figure 2E). Combined treatment with AG490 and LY294002 was most effective in reducing CPC number in CdM (Figure 2E). Incubation of CPCs in “Stattic”, an inhibitor specific to STAT3, confirmed the role of STAT3 activation in CdM-mediated effects on CPC proliferation (Supplemental Figure 2B). AG490 treatment also significantly reduced the number of CPCs incubated in serum-free medium, however, the effect of the AG490 was nearly equal to that of LY294002 treatment (Supplemental Figure 2C). Of interest, LY294002 but not AG490 significantly diminished CPC numbers in growth medium, indicating that the factors

promoting CPC growth in growth medium differed from the active factors in CdM that were STAT3-activating (Supplemental Figure 2C).

Insulin and Insulin-like growth factor 1 (IGF-1) bind to tyrosine kinase holoreceptors and promote cell survival and proliferation by signaling through the PI3K/Akt and Ras/MAP kinase pathways. Partial functional redundancy for Insulin and IGF-1 signaling is evidenced by signaling through IR/IGF-1R receptor heterodimers and bidirectional cross-talk by ligands and receptors [26]. Adult CPCs express the receptor for Insulin-like growth factor 1 (IGF-1) and injection of biotinylated IGF-1 nanofibers was shown to improve CPC grafts as well as endogenous myocardial regeneration after MI [27]. We observed that CSCs/CPCs did not proliferate in the absence of medium supplements containing Insulin (e.g. Insulin-Transferrin-Selenium [ITS] or fetal calf serum), even when in the presence of other mitogenic components from CSC/CPC growth medium such as LIF, EGF or bFGF (Supplemental Figure 3); this suggested that CdM from human stromal cells contained Insulin, IGF-1, or both.

CdM protects CPCs exposed to hypoxia

Because cells transplanted to the heart after MI may encounter hypoxic environments, we examined whether CdM could protect CPCs during exposure to 1% oxygen for 48 hrs *ex vivo*. Compared with cell survival in CPC growth medium (positive control; $100 \pm 3.01\%$), survival of CPCs in SFM was $48.63 \pm 21.13\%$, whereas that of CPCs incubated in MSC CdM was $87.515 \pm 3.97\%$ ($P < 0.01$ vs SFM), and in p75MSC CdM was $83.215 \pm 4.005\%$ ($P < 0.01$ vs SFM). Survival of CPCs incubated in 1x CdM from MSC donors and p75MSC donors was not significantly different ($n=4$ donors per cell type, $P = 0.206$). Of note, CdM produced from a different subpopulation of MSCs, CD133MSCs, did not

protect CPCs during culture under hypoxic conditions ($P=0.160$ vs SFM). Fibroblast CdM significantly protected CPCs ($70.79 \pm 1.91\%$, $P<0.05$ vs SFM), although both MSC CdM and p75MSC CdM were more protective than fibroblast CdM (each $P<0.01$ vs fibro). Inhibition of STAT3 abolished CdM-mediated protection of CPCs under hypoxic conditions (AG490, Figure 3B; Stattic, Figure 3C).

CPCs incubated in CdM retain their multipotent differentiation capacity

Differentiation assays were performed under normoxic conditions. Control CPCs cultured in CPC growth medium were negative for α -smooth muscle actin and von Willebrand Factor staining, whereas about 60% expressed α -sarcomeric actin (Supplemental Figure 4A, left and B). In contrast, clones of CPCs exposed to 1x CdM in culture for 4 days stained positively for α -sarcomeric actin, α -smooth muscle actin, and von Willebrand Factor (Supplemental Figure 4A and B). After 4 days in CdM, CPC-derived cells no longer expressed the CSC antigen, c-Kit, suggesting progress toward differentiation. Effects of intra-arterial infusion of p75MSC CdM after MI. Based on our results with CdM and cultured CPCs, we examined whether infusion of CdM after MI would increase the number of endogenous CPCs. MI was produced in C57bl6 mice by permanent ligation of the left anterior descending coronary artery (LAD). The following day, mice were randomized to receive treatment with intra-arterial infusion (left ventricle lumen) of 30x p75MSC CdM or vehicle (SFM). Mice from each group were euthanized at 1 day or 1 week after treatment. Hearts obtained 1 day after treatment were sectioned and processed for TUNEL and immunohistochemistry to detect c-Kit. TUNEL assays showed that CdM infusion significantly reduced cardiac apoptosis/necrosis (SFM, $17.2 \pm 8.1\%$; CdM, $3.7 \pm 2.2\%$; $P<0.05$, Supplemental Figure 5). Intra-muscular injection of porcine

MSCs from bone marrow, but not their conditioned medium, was reported to increase the number of c-Kit⁺ cells in the hearts of pigs after MI [28]. After intra-arterial infusion of CdM from human p75MSCs, we detected rare c-Kit⁺ cells in heart sections from both CdM- and SFM-treated mice. However, because c-Kit⁺ cell number was variable and did not appear differ between mice that received CdM or SFM, we did not quantify c-Kit⁺ cells. Biochemical assays for residual myocardial Creatine Kinase (CK) activity in left ventricular (LV) homogenates indicated that CdM- and SFM-treated mice had similar size infarcts at 1 week after MI (Anterior LV, SFM, 4.66 ± 0.40 ; CdM, 5.00 ± 0.43 , $P=0.21$; Posterior LV, SFM, 6.59 ± 0.32 ; CdM, 6.34 ± 0.33 , data expressed as IU CK/mg protein, mean \pm SD, $P=0.23$; SFM, n=6; CdM, n=5).

Priming of CSCs with CdM from p75MSCs improves graft success after MI

Based on our observations with p75MSC CdM and its ability to increase proliferation and survival of cultured CSCs/CPCs, we next determined whether prior exposure of CSCs to p75MSC CdM could foster the grafting of CSCs to the injured heart. MI was produced in Fischer rats by permanent ligation of the LAD. One day after MI, syngeneic GFP-positive rat CSCs were primed for 30 min on ice in p75MSC CdM (30x CdM, n=6 rats) or vehicle (SFM, n=5 rats). The chest wall was re-opened and rats were randomized to treatment with co-injections of CSCs/CdM or CSCs/SFM (125,000 cells/5 μ l injection, 2 sub-epicardial injections, 1 per border zone; please refer to Supplemental Figure 6 to view injection site). At 1 week and 1 month after MI, rats were euthanized and their hearts were processed as frozen serial-sections from apex to base. For each heart, we quantified GFP⁺ cells in the tissue section with the most GFP⁺ cells. At 1 week after MI, for 2 rats in the CSCs/SFM co-injection group, we did not detect GFP⁺ cells.

Furthermore, hearts from CSC/SFM rats with the highest level of engraftment contained less than 40 GFP⁺ cells/section (Figure 4A). In contrast, many sections from rats co-injected with CSCs/CdM contained several thousand GFP⁺ cells (Figure 4B-E). After priming of CSCs in p75MSC CdM, GFP⁺ cells grafted into sub-epicardial locations, proliferated (see Ki67 stain, Figure 4C), and migrated into zones with infarction (Figure 4C-E and Supplemental Figure 6). Furthermore, after 1 week, derivatives from CSCs primed in CdM engrafted into blood vessel walls as CD31-positive endothelial cells (Figure 4F). They also generated smooth muscle cells and myofibroblasts (smooth muscle alpha actin-positive, Figure 4G). Although they grafted into sub-epicardial locations after MI, GFP⁺ cells derived from vehicle-primed CSCs did not stain for Ki67 and had not entered myocardial tissue from sub-epicardial tissue at 1 week after MI and treatment (Figure 4A). At 1 month after MI, GFP⁺ cells were detected in infarct and border zones in multiple tissue sections and differentiated to CD31-positive endothelial cells. Whereas engraftment was extensive in 4 out of 4 animals that were injected with CSCs primed in p75MSC CdM, only 1 out of the 5 control animals had detectable cell engraftment at 1 month after MI. However, similar to control cell grafts at 1 week after MI, this animal had only a few GFP⁺ cells in the sub-epicardial tissue and none in the myocardial tissue. (Supplemental Figure 7A-C’’).

CdM contains human CTGF and bovine Insulin that activate CSCs/CPCs

To identify factors in p75MSC CdM that promoted the proliferation and survival of CPCs, we examined Affymetrix gene expression profiles from human p75MSCs that were freshly sorted from marrow aspirates and from p75MSCs cultured adherently for 2 passages [18]. Antibody blocking/neutralization studies for selected secreted factors were

carried out with 10x p75MSC CdM and CPCs exposed to hypoxic conditions (1% oxygen, 48 hrs). Under hypoxia, neutralizing antisera specific to human CTGF prevented CdM from protecting CPCs ($P < 0.001$, Figure 5A). Of interest, addition of human CTGF alone to SFM containing 1% BSA significantly induced p-STAT3 in CPCs (See inset, Figure 5B). By ELISA, 10x p75MSC CdM contained significantly more CTGF than did CD133MSC CdM (p75MSC, 1.29 ± 0.03 ng/ml; CD133MSC, 0.59 ± 0.13 ng/ml; $P < 0.01$), but did not differ in CTGF levels in comparison to MSC CdM (0.81 ± 0.14 ng/ml) or fibroblast CdM (1.1 ± 0.36 ng/ml). To our surprise, we did not detect IGF-1 in CdM, but we did detect Insulin. The amount of Insulin was not significantly different for CdMs from MSC, p75MSC, CD133MSC, or fibroblasts (about 2-3 ng/ml). Since the growth medium for MSCs and fibroblasts contained FCS (with bovine Insulin) and we did not detect human mRNA for Insulin by gene chip or by RT-PCR with cDNA from MSCs, we expect that the Insulin present in CdM was bovine in origin. Neutralizing antibodies specific to Insulin significantly reduced CdM-mediated protection of CPCs during hypoxia exposure, albeit not as much as did blocking CTGF (MTS assay[Abs⁴⁹⁰]: non-specific IgG, 0.378 ± 0.021 ; anti-Insulin, 0.270 ± 0.034 ; mean \pm SD, $n=4$, $P < 0.05$). Addition of recombinant human Insulin to CPCs induced p-Akt in a dose-responsive manner and increased also CPC survival and proliferation under hypoxic conditions (Figure 5C). Notably, CPC protection assays with human Insulin or IGF-1 alone (30 ng/ml, each) demonstrated that they were equivalent in their ability to rescue CPCs exposed to hypoxia (control [SFM with 1% BSA]: 9004 ± 12 cells; Insulin: $13,507 \pm 1,473$ cells; IGF-1, $14,894 \pm 559$ cells; mean \pm SD, $n=3$, $P = 0.24$).

CTGF (CCN2, IGFBP8) consists of 4 domains and the C-terminal (4th) domain alone was reported to increase cell adhesion and proliferation [29,30]. In experiments with recombinant peptides, we found that combined treatment with human C-terminal CTGF (CTGF-D4) and Insulin had synergistic effects on CPC survival and proliferation under hypoxic conditions. For example, CTGF-D4 or Insulin alone (1 ng/ml in SFM, each) did not protect CPCs against 48 hr of hypoxia (Figure 5D). In contrast, addition of both CTGF- D4 and Insulin to SFM (1 ng/ml, each) provided significant protection of CPCs against hypoxia ($P < 0.05$, Figure 5D). CTGF-D4/Insulin-mediated protection of CPCs was enhanced by including 1% BSA as a carrier ($P < 0.01$, Figure 5D). Clones of CPCs exposed to CTGF-D4 (3ng/ml) and Insulin (30ng/ml) in culture for 4 days stained positively for α -sarcomeric actin, α -smooth muscle actin, and von Willebrand Factor at ratios similar to those observed after 4 day CPC differentiation in 1x CdM (Supplemental Figure 4B). These results indicated that the multipotency of CPCs was retained after exposure to CTGF-D4 and Insulin.

A defined combination of CTGF-D4/Insulin promotes CSC grafts after MI

Having observed synergistic protective effects after CTGF-D4/Insulin treatment of cultured CPCs exposed to simulated ischemia, we hypothesized that a priming mixture based on the CTGF-D4/Insulin ratio found in 30x p75MSC CdM would promote CSC engraftment when using to prime CSCs. One day after MI, GFP CSCs were incubated on ice with SFM containing 1% BSA, CTGF-D4 (3 ng/ml), and Insulin (30 ng/ml), or with vehicle (SFM with 1% BSA) for 30 min prior to co-injection into border zone areas of rats randomized to treatment (125,000 cells/5 μ l injection, 2 sub-epicardial injections, 1 per border zone). As before, all rats were euthanized 1 week after MI and their hearts

were processed as serial sections. Whereas few rats that received co-injections of CSCs/vehicle had detectable GFP⁺ cells after 1 week (1/7 rats, Figure 6A and A'), all rats that received CSCs/CTGF-D4/Insulin exhibited a level of engraftment consistent with results obtained by priming with 30x p75MSC CdM (5/5 rats, Figure 6B-C'). Similar to CSCs primed in CdM, CSCs primed in CTGF-D4/Insulin grafted into sub-epicardial locations, proliferated, and provided GFP⁺ CSC derivatives that migrated into host myocardium, reaching areas of infarction with few remaining viable myocytes (Figure 6B'-C'). At one month after MI, 4 out of 5 rats that received CSCs primed in CTGF-D4/Insulin exhibited widespread engraftment cells of GFP⁺ derivatives. Similar to priming of CSCs in CdM, CTGF-D4/Insulin priming facilitated survival, migration and differentiation of CSC derivatives to CD31-positive and smooth muscle actin positive cells in infarct area at 1 month (Supplemental Figure 7D-E').

Discussion

Pre-conditioning strategies that expose cells to hypoxia, heat-shock, protein/peptide growth factors, chemicals and drugs have been shown to increase the survival of adult stem/progenitor cells, ES cells, and ES cell-derivatives after transplantation to the heart [31-36]. Although significant in terms of effect, many reported strategies result in modest cell grafts that are unlikely to repair the large tracts of necrotic tissue generated after MI. Our results indicate that short-term priming in p75MSC CdM or CTGF-D4/Insulin may boost graft success for clinical application of CSCs and perhaps also cultured stem/progenitor cells derived from other tissues or sources.

Priming in IGF-1 was reported to improve the survival of cardiac cell grafts with adult and embryonic stem cells [36]. We found that adult rat CPCs were protected equally-well by Insulin or IGF-1 during hypoxia in culture. Although human p75MSC CdM did not have detectable IGF-1, we found that it contained sufficient residual bovine Insulin to significantly improve CPC survival under hypoxic conditions. CdM contains Insulin because MSCs internalize fetal calf serum components such as albumin, IgG, and Insulin from their growth medium. Even after multiple washes, they can release some components back into the base medium used for CdM production [37].

CTGF is a secreted “matricellular” protein with multiple functions in mammalian development and tissue remodeling/repair after injury, including angiogenesis and fibrosis [38-41]. During pancreatic development, CTGF promotes the proliferation of beta cell progenitors in islets [42]. Cardiac expression of CTGF increases significantly after MI and it is expressed by interstitial fibroblasts and cardiac myocytes [43]. By interacting with the extracellular matrix, integrins and several cell surface receptors (e.g. LRP-1, LRP-6, TrkA), CTGF mediates numerous cellular functions including: adhesion, proliferation, migration, differentiation, and survival [44-46]. To regulate coincident processes after injury such as angiogenesis and fibrosis, CTGF physically associates with numerous other secreted proteins including VEGFA, Slit3, von Willebrand Factor, PDGF-B, BMP-4, IGF-1, IGF-2, TGF alpha and TGF beta[30, 38-41]. Although we do not identify them here, it will be of great interest to determine which CTGF binding partners control graft success.

CTGF controls fibrosis in multiple tissues after injury, in part, by interacting with TGF beta, IGF-1 or IGF-2 and promoting the differentiation of fibroblasts into

myofibroblasts. The N-terminal (1st) and 2nd domains of CTGF interact with IGFs and TGF beta or BMP4, respectively. Notably, due to its numerous binding partners, the effects of CTGF are context-dependent. In the presence of cellular mitogens such as EGF, CTGF does not induce fibrosis, even when pro-fibrotic mediators like TGF beta or IGF-2 are present [40,41]. Importantly, the CTGF-D4/Insulin priming method reported here is unlikely to promote myofibroblast differentiation or fibrosis from transplanted CSCs as CTGF-D4 is known to promote cell adhesion and proliferation, but lacks N-terminal functions in fibrosis [29, 30, 40, 41].

To date, clinical trials or animal models designed to graft cells to the heart after MI primarily administer cells intravenously, intra-arterially, or intra-muscularly. Here we developed a tangential injection method to deliver CSCs to the sub-epicardial space lying outside and adjacent to the injured myocardium. In addition to priming CSCs in p75MSC CdM or CTGF-D4/Insulin, we found that the border zone sub-epicardial space was important for cell injections. Notably, CSCs injected into the sub-epicardial space of healthy hearts, or, into the sub-epicardial space lying above normal, uninjured tissue in hearts with MI did not proliferate or migrate in the manner observed for border zone injections after MI (data not shown). These observations indicate that the border zone sub-epicardial environment may itself contain factors that promote cardiac graft success. Whereas our injections were performed in an open-chest fashion, for a less-invasive procedure in patients it may be possible to use catheter-based cell injection to reach the sub-epicardial space through the myocardium.

Multiple tissue-specific cell types express integrins and cell surface receptors known to interact with CTGF and Insulin, including stem/progenitor cells of adult tissues.

In addition to improving cell grafts for the heart, we are hopeful that priming with CdM from stromal progenitors or with CTGF-D4/Insulin will promote graft success for other solid tissues after injury.

Materials and Methods

Preparation of human stromal progenitor cells and fibroblasts

Human MSCs, p75MSCs and CD133MSCs were prepared with protocols approved by an Institutional Review Board. For the experiments outlined here, we used banked vials of stromal progenitor cells that were characterized previously for the same human donors [18]. Human dermal fibroblasts were obtained from the cell bank of the Tulane Center for Gene Therapy; they were isolated from skin punch biopsy samples under a protocol that was approved by an Institutional Review Board [18,47].

Preparation of serum-free conditioned medium (CdM). Passage 4 to 8 human MSCs, p75MSCs, CD133MSCs, or dermal fibroblasts were cultured in 150 cm² dishes with complete culture medium [18]. For the present study, the various human cell types were plated at 1000 cells/cm² for seeding, underwent about 3 population doublings per passage, and were frozen down at each passage. To generate CdM, cells at 80 to 90% confluence were washed twice with PBS and incubated with 20 mls of fresh serum-free α -MEM in standard conditions without any supplements or growth factors for 48 hrs. CdM was then collected, filtered, and stored at – 80 C°. For some experiments, CdM was concentrated up to 10- or 30-fold with the use of a Labscale™ TFF diafiltration system (Pellicon XL 5 kDa cut-off filters, Millipore, Bedford, MA).

Isolation and culture of adult rat cardiac stem/progenitor cells

Adult CSCs were isolated from the ventricles of Fischer 344 rats and labeled with retroviral vector for GFP [21]. CSCs were cultured as floating spheres in DMEM/F12 supplemented with bFGF (10 ng/ml), EGF (20 ng/ml), LIF (10 ng/ml) and ITS. To grow adherent CPCs, CSCs were plated at 500 cells/cm² and cultured in CSC medium supplemented with 2% FBS (CPC growth medium).

Short-term priming of CSCs

CSCs were cultured as spheres in serum-free CSC growth medium. CSC spheres were trypsinized and centrifuged at 1000 x g for 8 min. After re-suspension in 1x PBS, cells were passed through a 40 micron filter to isolate single CSCs (cell strainer, Fisher Scientific). Cells were counted on a hemocytometer, centrifuged again, and re-suspended in 30x p75MSC CdM or Alpha-MEM (CdM vehicle control), or CTGF-D4 (3ng/ml)/Insulin (30ng/ml)/1% BSA in α -MEM, or 1% BSA in α -MEM (vehicle control for recombinant peptides). CSCs were incubated in the above conditions for 30 minutes on ice prior to sub-epicardial injection.

Myocardial infarction surgery and CSC transplantation in rats

Fischer 488 rats (males, 7 weeks of age) were weighed, shaved, anesthetized under 4% isoflurane, and endotracheally-intubated. Rats were ventilated at a respiration rate of 65 beats per min under a peak inspiration pressure of 15 cm H₂O (Kent Scientific). Body temperature was maintained at 37°C with a heating pad (Gaymar). Through a dermal incision, a blunt dissection of the fascia was performed and the intercostal muscles were separated. The heart was exposed by retraction of the pericardium to expose the LAD.

The LAD was occluded with a 6-0 nylon suture and occlusion was confirmed by blanching of the anterior free wall of the LV. The animals were allowed to recover off the ventilator.

After 24 hours, rats were re-intubated, ventilated, and the chest wall was re-opened. Hearts were exposed to reveal the border zones of the infarct. For each rat, we performed 2 sub-epicardial injections of CSCs (5 μ l each, one per border zone) with a 30 gauge Hamilton syringe. The needle was introduced tangentially to the wall of the LV and with the bevel facing upward. The syringe was advanced only as far as the bevel edge to access the sub-epicardial surface of the heart and so as not target the underlying myocardium of the LV. After the injections, the chest wall was closed and rats recovered for 7 days or 1 month prior to euthanization.

An expanded Methods section with detailed descriptions for isolation of human bone marrow-derived MSCs and p75MSCs, isolation and culture of adult rat cardiac fibroblasts, cell culture in CdM and evaluation of cell number, immunocytochemistry, DNA replication assays, immunoblotting, ELISAs for IGF-1, Insulin, and CTGF, MI surgery in mice, infusion of p75MSC CdM after MI in mice, TUNEL Assay, Creatine Kinase (CK) activity assay, immunohistochemistry, and statistical analysis is available in the online-only Data Supplement.

Acknowledgments

This work was supported in part by NIH grants HL077570 and HL085210 (to J.L.S.). Special thanks to Alexander Aronshtam, Ph.D., Keara McElroy-Yaggy, Patricia Baumann, Dagnija Neimane, and Calvin Yang for technical assistance.

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Supplementary Material

Methods

Isolation of human bone marrow-derived MSCs and p75MSCs. To obtain MSCs, bone marrow aspirates were taken from the iliac crest of healthy adult donors. Mononuclear cells were isolated with the use of density gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech) and resuspended in complete culture medium consisting of Alpha-MEM (GIBCO/BRL, Grand Island, NY); 17% FBS (Atlanta Biologicals, Norcross, GA); 100 units/ml penicillin (GIBCO/BRL); 100 µg/ml streptomycin (GIBCO/BRL); and 2 mM L-glutamine (GIBCO/BRL). Cells were plated in 20 ml of medium in a 150 cm² culture dish and incubated in a humidified incubator (Thermo Electron, Forma Series II, Waltham, MA) with 95% air and 5% CO₂ at 37°C. After 24 h, nonadherent cells were removed. Adherent cells were washed twice with PBS and incubated with fresh medium. The primary adherent cells were cultured and propagated. To obtain p75MSCs or CD133MSCs, bone marrow stem/progenitor cells were isolated by MACS using antibodies against the p75LNGFR or CD133 (Prominin 1). Freshly isolated bone marrow mononuclear cells from the Ficoll gradient were resuspended in 0.4 ml of PBS containing 0.5% bovine serum albumin and 2 mM EDTA. After adding mouse anti-human antibody conjugated to magnetic beads (CD271, CD133; Miltenyi Biotech, Auburn, CA), the sample was incubated for 30 min at 4°C, and then applied to a magnetic column (LS Column; Miltenyi Biotech). The bound fraction was eluted with 5 ml of MACS buffer and the cells were concentrated by centrifugation at 1000 x g for 8 min. After re-suspension, the entire isolate was cultured in complete culture medium.

MSC-like cells appeared as small colonies after about 1 week, and the cells were expanded.

Isolation and culture of adult rat cardiac fibroblasts. Procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Ventricular fibroblasts were isolated from hearts of adult Sprague-Dawley rats. The hearts were minced and enzymatically-dissociated into single cell suspension. Non-myocytes were separated by discontinuous density gradient centrifugation and cultured in DMEM/F-12 supplemented with 10 % FCS. Passage 2 cells were used for experiments. Cell culture in CdM and evaluation of cell number. Adult rat CPCs and cardiac fibroblasts were plated at 500 cells/cm² and cultured in their respective growth mediums. Three days after plating, the medium was removed, the wells were washed twice with PBS, and the cells were then exposed to CdM or to fresh serum-free medium (Alpha-MEM). For time course proliferation studies, CdM and serum-free medium were changed every 2 days. In signal transduction inhibitor studies, we used the following pharmacological inhibitors: AG490, inhibitor of Jak2/STAT3 pathway; Stattic, inhibitor of STAT3; LY294002, inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt pathway; and PD98059, extracellular signal-regulated kinase (ERK) inhibitor. All of the inhibitors were purchased from Calbiochem (Darmstadt, Germany) and were dissolved in dimethyl sulfoxide (DMSO). CPCs were cultured in CdM with the inhibitors or with the equivalent volume of DMSO as a control for 48 hrs. In cell protection studies, 3 days after plating, medium was replaced with either the CdM or serum-free medium and the cells were exposed to hypoxia in a specialized incubator (1% oxygen) for 48 hrs. The hypoxia

incubator was a model that measured both CO₂ and O₂ (Thermo Electron, Forma Series II, model 3130). Oxygen was maintained at 1% by the injection of nitrogen gas and was monitored continuously.

Cell numbers were quantified by the fluorescent labeling of nucleic acids (CyQuant dye; Molecular Probes, Carlsbad, CA) and with a microplate fluorescence reader (FLX800; Bio-Tek Instruments Inc., Winooski, VT) set to 480 nm excitation and 520 nm emission. Each experiment was repeated a minimum of 3 times.

Immunocytochemistry. CPCs were fixed with 4% paraformaldehyde in 1x PBS. Non-specific binding was limited by 1 hour incubation in PBS containing 5% goat serum and 0.4% triton X-100. Primary antibodies were incubated overnight at 4°C. After washing 3x 5 min with PBS, secondary antibody that was diluted 1:1000 (Alexa 594, Molecular Probes) was applied for 1 h at room temperature (RT). After 3x 5 min washes, slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Epifluorescence images were taken using a Leica DM6000B microscope equipped with a CCD camera (Leica DFC350Fx) and FW4000 software. The primary antibodies for immunocytochemistry were as follows: phospho-STAT3 (1:50, Tyr705, #9131; Cell signaling, Danvers, MA); α -sarcomeric actin (1: 500, clone 5C5, #A2172; Sigma); α -smooth muscle actin (1: 800, clone 1A4, #A5228; Sigma); and von Willebrand factor (1: 100, clone 21-43, # AB3442; Chemicon, Temecula, CA). For quantification of differentiation, cells positive for α -sarcomeric actin, α -smooth muscle actin and von Willebrand factor and total cells were counted at least in three fields per slide. The percentage of positive cells was calculated for each slide (n=3 in each group).

DNA replication assay. Three days after the plating, CSCs were cultured in growth medium, CdM or serum-free medium for 24 h, and BrdU (BD Biosciences) was added at a final concentration of 10 μ M. Immunocytochemistry was carried out with BrdU antibody (1:100 clone BU33, # B8434; Sigma) and BrdU-positive cell numbers were quantified as above.

Immunoblotting. Cells were lysed in a buffer that consisted of 0.1% sodium dodecyl sulphate (SDS) and complete protease inhibitor cocktail (Roche, Basel, Switzerland) in PBS. Protein concentration was determined by the DC protein assay (Biorad, Hercules, CA). Twenty μ g of protein was separated by SDS-PAGE. After electrophoresis, the gels were electroblotted to polyvinylidene difluoride (PVDF) membranes. All electrophoresis and electroblotting used Novex reagents and systems (Invitrogen, Carlsbad, CA). The blots were blocked for 1 h at RT in 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBST), washed 3×5 min in PBST, and incubated in primary antibodies in PBST with 5% BSA overnight at 4°C. After 3×5 min washes in PBST, the blots were incubated in secondary antibody conjugated to horseradish peroxidase conjugate (1: 2000, Sigma) in PBST for 1 h at RT. Unbound secondary antibody was removed and positive bands were detected with a chemiluminescent reaction. The primary antibodies for immunoblotting were Ki67 (1: 200, clone SP6, Abcam, Cambridge, MA); p-STAT3 (Tyr705, 1: 1000), total-STAT3 (1: 1000, #4904, Cell Signaling), p-Akt (Ser 473, 1:1000, # 9271, Cell Signaling), total- Akt (1:1000, # 9272, Cell Signaling), Insulin (1:1000, # I2018, Sigma); and β -actin (1: 5000, clone AC-15, # A5441, Sigma).

ELISAs for IGF-1, Insulin, and CTGF. For assay of human IGF-1, we used 1x p75 CdM and commercial ELISA reagents with the manufacturer's protocol (# DY291, R and D

Systems). For assay of Insulin and CTGF, high protein-binding plates were incubated with 1 or 10x p75 CdM overnight at room temperature to capture antigens from CdM. Wells were then washed with mild detergent (0.05% Tween-20 in PBS) followed by blocking with 1% BSA in PBS for 1 hour. After blocking buffer was thoroughly washed off from the wells, samples were incubated with 100 μ l of biotin-conjugated polyclonal antibody to CTGF at 5 μ g/100 μ l (#P252Bt, Peprotech) for 2 hours at room temperature. Polyclonal mouse anti-Insulin antibody (Santa Cruz) was incubated for 2 hours at room temperature, followed by 3 washes with wash buffer. The wells were then incubated in anti-mouse biotin conjugated IgG (B7264, Sigma Aldrich) for 2 hours at room temperature. After washing in wash buffer 3 times, samples for CTGF and Insulin ELISA were incubated in Streptavidin conjugated HRP (1:2000) for 2 hrs at room temperature, followed by washing and addition of 100 μ l of substrate ABTS (Thermo Scientific; #37615) for 20 minutes. Absorbance was measured (450 nm) on a Synergy HT plate reader.

Myocardial infarction surgery in mice. Male mice at 9-10 weeks of age underwent permanent ligation of the Left Anterior Descending Coronary Artery (LAD) to induce myocardial infarction (C57bl6 mice, Taconic, Hudson, NY). Mice were not included in the study if they did not survive the initial MI surgery, did not achieve a successful MI (blanching observed at time of treatment), or died during treatment application. Following all procedures, mice were given analgesia (buprenorphine, 0.05-0.1 mg/kg i.p.) and monitored for signs of distress until termination of the study. All procedures were done in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Vermont.

For permanent LAD ligation surgery, mice were anesthetized with 2-4 % Isoflurane, shaved, weighed, intubated, and then maintained for the duration of the procedure on a sterile surgical field with the use of a mechanical ventilation system (MiniVent, Harvard Apparatus, Holliston, MA). Throughout the surgery and during the recovery period, body temperatures were maintained with a heated water pad system (Gaymar T-Pump TP-500, Gaymar Industries, Orchard Park, NY). Viewing the chest through a dissecting microscope (Stemi 2000-C, Carl Zeiss MicroImaging, Thornwood, NY) a dermal incision was made, the underlying fascia were removed, and the thoracic musculature was retracted to expose the left ribcage. Next the intercostal muscles were retracted and the outer (parietal or visceral) pericardium was removed to expose the LAD. The LAD was then ligated (2.0-3.0 mm from left atrial apex) with 8.0 nylon suture (Henry Schein, Melville, NY) and blanching within the myocardium of the left ventricle was noted. The intercostals were rejoined with a 6.0 nylon suture (Henry Schein), the lungs were reinflated, and overlying dermis rejoined with a 6.0 nylon suture. All mice were recovered to an ambulatory state prior to any subsequent treatment procedure. Survival after the MI surgery was > 90%. Sham-operated mice underwent all procedures except that the suture was placed under the LAD but was not ligated.

Infusion of p75MSC CdM after MI in mice. To evaluate p75 CdM treatment in an unbiased manner, all animals were randomized to treatment (after LAD ligation).

Following 24 hour recovery of an animal after the first surgery, the mouse was then again anesthetized, intubated, and the chest opened. Once the intact suture and area of blanching were confirmed, 30x p75 CdM (200 uL) or vehicle (Alpha-MEM, 200 uL)

warmed to 37°C was delivered to the entire cardiovascular arterial tree by injecting the solution into the lumen of the left ventricle (LV). Injections were performed slowly (over a period of 1 minute) with a 30.5 gauge needle inserted below the great cardiac vein (LV apex) at an angle 45° to the myocardium. Following treatment with either CdM or vehicle, the needle was removed and the intercostals were rejoined using 6.0 chromic gut suture (Ethicon, Johnson and Johnson, Inc., Livingston, UK), lungs then reinflated, and overlying dermis rejoined with 6.0 nylon suture. All mice were then recovered to an ambulatory state and transferred to the vivarium for the remaining duration of the experiment.

For CK assays, left ventricular tissue was dissected away from the atria and the aorta, further separated into anterior LV and posterior LV/septum, and immediately snap frozen by submersion of cryovials in liquid N₂. The LV tissues were maintained at -80°C until the day of the CK assay (see below).

TUNEL Assay. TUNEL was performed as reported previously.¹ Quantification of TUNEL- positive cells within zones of infarction was performed in an unbiased fashion by a viewer that was blinded to slide (sample) identity. Cells were counted with Image Pro Plus Software as reported previously [1].

Creatine Kinase Assay. The remaining creatine kinase (CK) activity in left ventricular tissues was assessed to determine the extent of infarction in mouse hearts as reported previously [2,3]. The loss of CK activity directly reflects the loss of viable myocardium

after MI. The percentage of left ventricle with infarction was calculated based on observed total LV CK activity (IU/mg protein) in left ventricles of normal hearts without infarction. The percent of MI = $100 \times [\text{NL CK} - \text{LV CK}] / \Delta$, where NL CK is the amount of CK in tissue from normal LV (IU/mg of soluble protein), LV CK is total remaining CK activity in the LV after MI (IU/mg soluble protein), and Δ is the difference between the amount of CK in normal zones of myocardium and in zones of myocardium with infarction.

Immunohistochemistry. Rats were euthanized under isoflurane, their hearts harvested and washed in PBS to remove remnant blood. Hearts were fixed in 4% paraformaldehyde overnight and equilibrated in 15 and 30% sucrose consecutively for cryoprotection. After mounting in OCT (Tissue-Tek), serial sections were performed from apex to base at 20 microns (Leica CM1800 Cryostat) and sections were mounted on glass slides. Slides were dried at 37°C and washed twice in 1x PBS. Primary antibodies were against Ki67 (clone SP6, 1:100; Abcam), CD31, (1:50, #555026, BD Biosciences), and smooth muscle alpha actin (1:500, Sigma). Primary antibodies were detected with secondary antibodies conjugated to Alexa 574 (1:2000). Slides were mounted in Vectashield with DAPI (Vector Labs). Sections were imaged by epifluorescence deconvolution microscopy (Leica DM6000B; Leica) with Leica FW4000 software.

Statistical analysis. Comparisons of parameters among the three groups were made with one-way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test. Comparisons of parameters between two groups were made by unpaired Student's t-test. $P < 0.05$ was considered significant.

References for Supporting Information:

1. French, CJ, Spees, JL, Zaman, AK, Taatjes, DJ, Sobel, BE. The magnitude and temporal dependence of apoptosis early after myocardial ischemia with or without reperfusion. *FASEB J.* 2009;23:1177-1185.
2. Kjekshus, JK, Sobel, BE. Depressed myocardial creatine phosphokinase activity following experimental myocardial infarction in rabbit. *Circ Res.* 1970;27:403-414.
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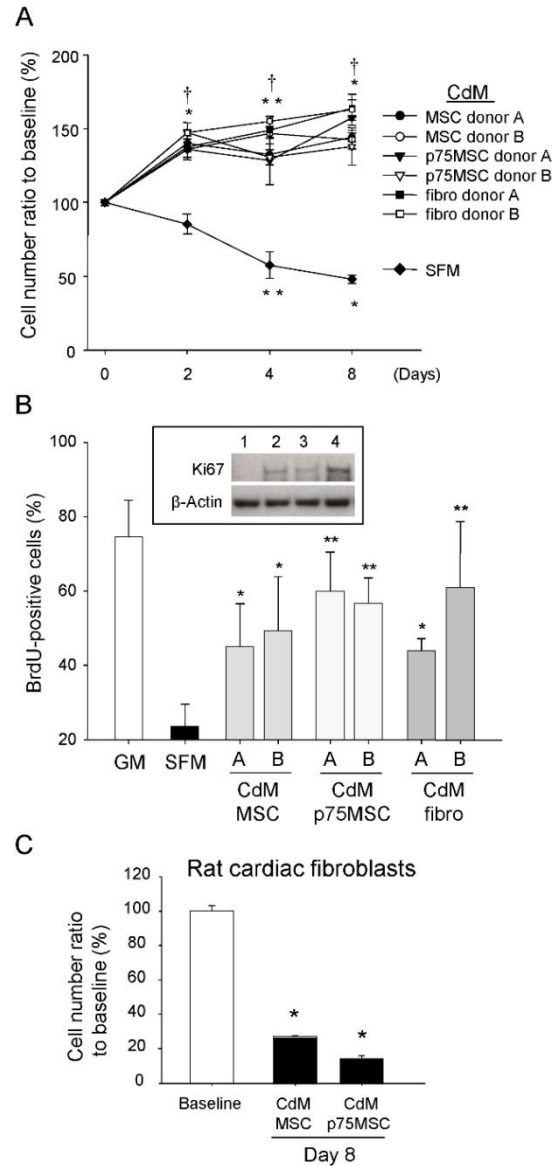


Figure 2- 1. Human stromal cell CdM induces proliferation of adult rat CPCs

(A) Time course changes in the numbers of CPCs treated with CdM or SFM. CdM was assayed from 2 different donors for each cell type. Control cell number (48,896 cells) was regarded as 100%. Data are mean \pm SEM, $n = 3$ to 7. *, $P < 0.0001$ vs baseline; **, $P < 0.01$ vs baseline; †, $P < 0.0001$ vs SFM. (B) Quantification of BrdU-positive CPCs after immunocytochemistry. Data for 2 human donors are shown for each cell type [donor A and donor B]. Inset, Immunoblot for Ki67 in CPCs (molecular weight, 359 kDa: lane 1, SFM; lane 2, 1x MSC CdM; lane 3, p75MSC CdM; lane 4, 1x fibroblast CdM). *, $P < 0.05$ vs SFM; **, $P < 0.01$ vs SFM. (C) MSC CdM does not support cardiac fibroblast proliferation. Control cell number (34,606 cells) was regarded as 100%. For B and C, data are mean \pm SEM, $n = 3$. *, $P < 0.0001$ vs baseline. CdM, conditioned medium. SFM, serum-free α -MEM. GM, CPC growth medium (CSC medium with 2% FBS).

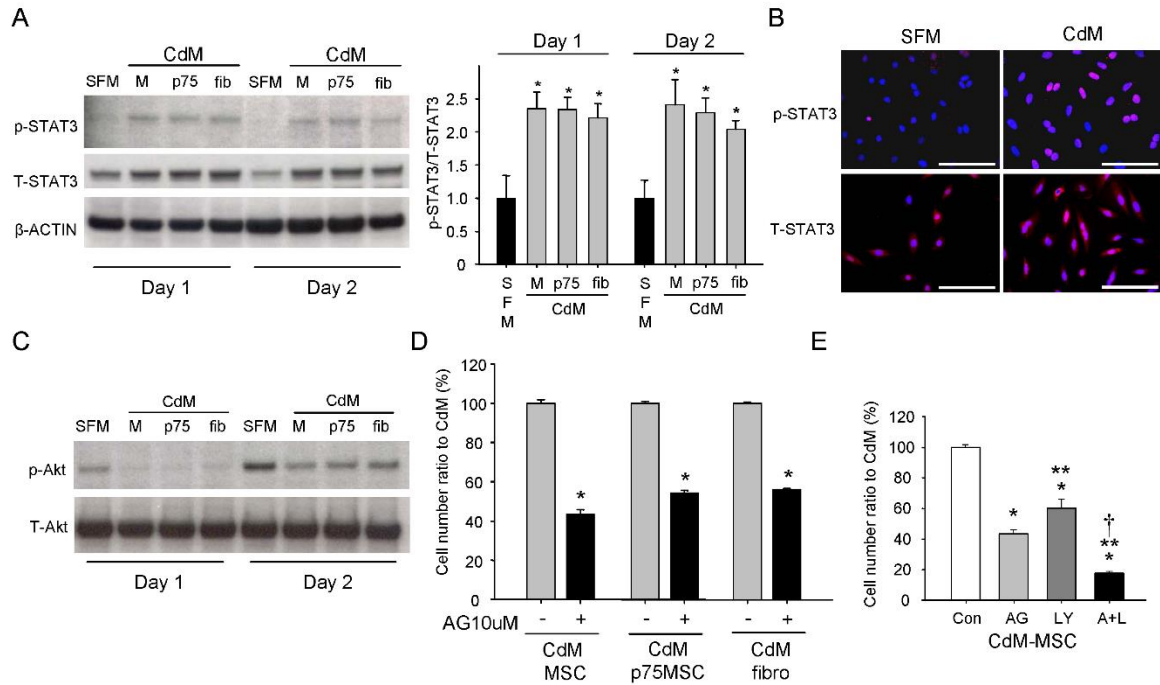


Figure 2- 2. STAT3 and Akt activation in CPCs treated with 1x CdM

(A) Left, Immunoblotting for phosphorylated STAT3 (p-STAT3) and total STAT3 (T-STAT3) in CPCs (molecular weight, 86 kDa). Beta-actin levels indicate loading. Right, Quantification of p-STAT3 levels (n = 3). The corrected values in SFM on day 1 and 2 were designated as “1”. *, P < 0.05 vs SFM. (B) Immunocytochemistry for p-STAT3 and T-STAT3. Note: p-STAT3 localizes to CPC nuclei (blue, DAPI). Scale bars = 100 μM. (C) Immunoblotting for phosphorylated Akt (p-Akt) and total Akt (T-Akt) in CPCs (molecular weight, 62 kDa). (D) Inhibitory effect of AG490 (10 μM) on CPC growth and survival in stromal cell CdM for 48 hrs. Control cell numbers (121,863 cells in MSC CdM, 115,342 cells in p75MSC CdM, and 118,682 cells in fibro CdM) were regarded as 100%. n = 3 replicates for 1 human donor per cell type. *, P < 0.0001 vs control. (E) Inhibitory effects of AG490 (10 μM) and LY294002 (10 μM) on CPCs incubated with 1x CdM for 48 hrs. Control cell numbers (121,863 cells) were regarded as 100%. Data are mean ± SEM, n = 3 to 6 replicates. *, P < 0.0001 vs control; **, P < 0.01 vs AG; †, P < 0.05 vs LY. Con: control, DMSO. AG: AG490, Jak2/STAT3 pathway inhibitor. LY: LY294002, inhibitor of PI3K/Akt pathway. A+L: AG490 + LY294002.

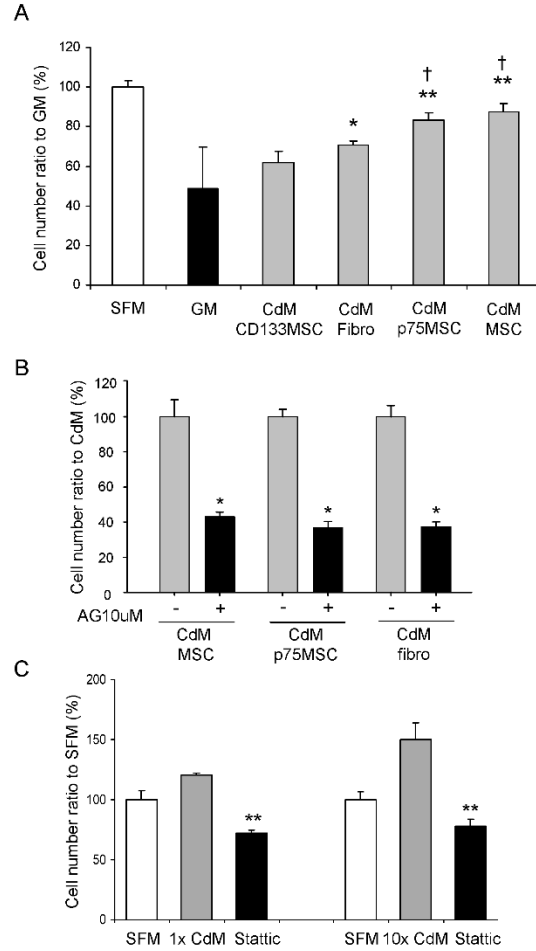


Figure 2- 3. Protective effect of human stromal cell CdM on rat CPCs exposed to chronic hypoxia (1% oxygen for 48 hrs)

(A) CPC numbers in GM, SFM and CdM after exposure to chronic hypoxia. Control cell number (58,616) was regarded as 100%. Data are mean \pm SEM. CdM donors assayed for each cell type; n=3 (CD133MSC, Fibro), n=4 (p75MSC, MSC). *, $P < 0.05$ vs SFM; **, $P < 0.01$ vs SFM; †, $P < 0.01$ vs CD133MSC CdM. (B) STAT3 inhibition with AG490 (10 μ M) blocks CPC protection conferred by CdM during hypoxia. Control cell number (56,559 cells in MSC CdM, 92,120 cells in p75MSC CdM, and 74,511 cells in fibro CdM) was regarded as 100%. Data are mean \pm SEM, n = 3 replicates for 1 human donor per cell type. *, $P < 0.0001$ vs CdM. AG: AG490, Jak2/STAT3 pathway inhibitor. (C) Survival of CPCs in p75MSC CdM and under hypoxic conditions for 48 hrs is dependent on signaling through STAT3 and abolished by incubation with the STAT3-specific inhibitor, “Stattic” (10 μ M). n = 3 replicates for 1 human donor. **, $P < 0.001$ vs DMSO vehicle with 1x or 10x CdM. CdM, conditioned medium. SFM, serum-free α -MEM. GM, CPC growth medium (CSC medium with 2% FBS).

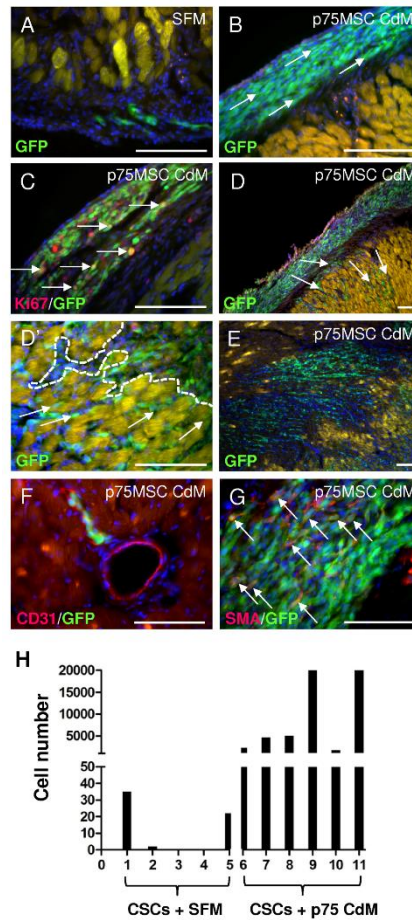


Figure 2- 4. Priming of adult rat CSCs with human p75MSC CdM markedly improves CSC graft success 1 week after MI

At 1 day after MI, CSCs were primed in 30x CdM or vehicle (SFM) for 30 min on ice prior to co-injection (2 sub-epicardial injections per rat, 1 per border zone). (A) Image of the largest cell graft for control rats (n = 5), at 1 week after MI and injections of CSCs/SFM. Yellow autofluorescence indicates host-derived myocytes. (B-D) Images of sub-epicardial grafts from three different rats, 1 week after MI and injections of CSCs/CdM (n = 6). (C) Ki67 staining (red) of proliferating GFP⁺ CSC derivatives in sub-epicardial tissue after CSC/CdM injections. (D, D') For hearts treated with CSCs/CdM, GFP⁺ cells were observed to migrate between apparently healthy cardiac myocytes in order to reach distant zones of necrotic myocardium with infarction. White arrows in (D) indicate change in CSC orientation during migration from sub-epicardium into myocardium after MI; compare to orientation in (B). The dashed white line in D' indicates edge of infarction. (E) After CdM-priming, GFP⁺ CSC derivatives migrated into areas of necrosis with few remaining viable myocytes. (F) CdM-primed CSC derivatives differentiate into CD31-positive (red) vascular endothelial cells to repair blood vessels. (G) CdM-primed CSC derivatives differentiate into smooth muscle alpha actin-positive (SMA, red) smooth muscle cells and myofibroblasts (arrows indicate co-localizations with GFP). (H) Quantification of GFP⁺ cells from individual tissue sections with the most engraftment in hearts that received CSCs/SFM (rats 1 - 5) or CSCs/CdM (rats 6 - 11). Note: We stopped counting after 20,000 GFP⁺ cells for two CSC/CdM-treated rats, but observed thousands of additional GFP⁺ cells. Images A, B', C, D', F, G: Scale bars = 100 μ M; D, E: Scale bars = 50 μ M.

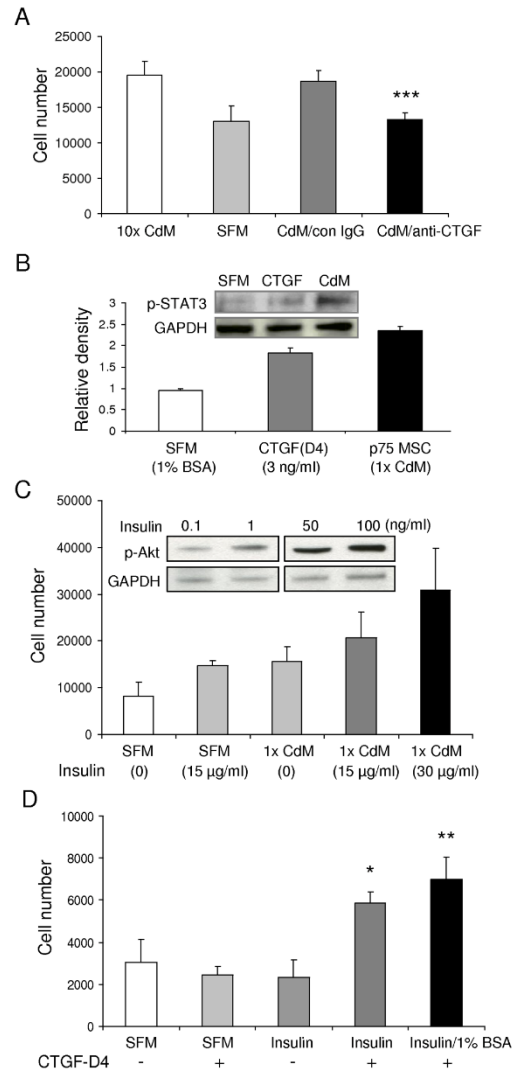


Figure 2- 5. CTGF and Insulin are key factors present in p75MSC CdM that promote the survival and proliferation of CPCs

(A) Incubation of 10x p75MSC CdM with antisera specific to human CTGF ablates its ability to protect CPCs during 48 hrs of hypoxia. Non-specific IgG (con IgG) or anti-CTGF was added to separate aliquots of CdM (10 µg/ml, each). ***, $P < 0.001$ vs. con IgG. (B) CTGF (3 ng/ml, 30 min incubation) induces p-STAT3 in CPCs compared with incubation in vehicle (1% BSA). Levels of p-STAT3 after 30 min incubation in 1x CdM are shown for comparison. Inset, Representative blot. (C) Effects of Insulin on survival and growth of CPCs under normoxic conditions. Inset, Increasing Insulin concentration has dose-responsive effect on p-Akt levels in CPCs (30 min incubation). (D) Dual incubation of CPCs with C-terminal domain 4 peptide (CTGF D4) and Insulin (1 ng/ml, each) has synergistic effects on CPC survival during 48 hrs of hypoxia. *, $P < 0.05$ vs SFM; **, $P < 0.01$ vs SFM. For A-D, $n = 3-5$. CdM, conditioned medium. SFM, serum-free α -MEM.

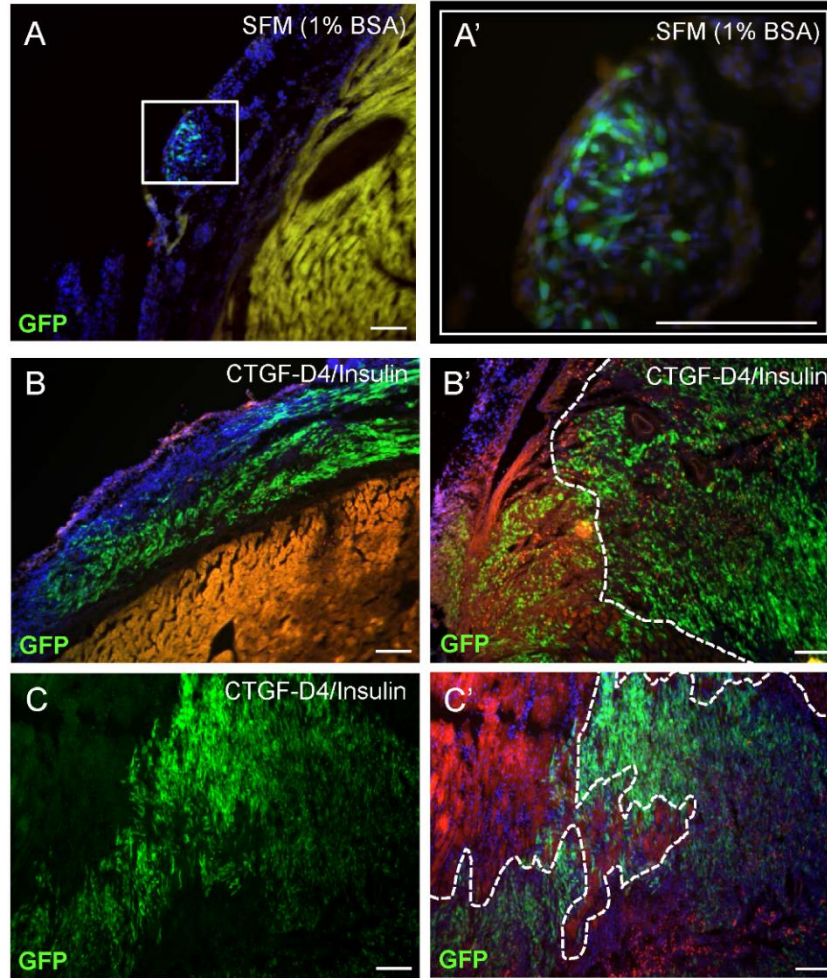


Figure 2- 6. Priming of cultured CSCs in CTGF-D4 and Insulin promotes graft success after MI

(A, A') Few control rats (1/7) injected with CSCs primed for 30 min in vehicle (SFM with 1% BSA) had detectable GFP⁺ cells at 1 week after MI and sub-epicardial injections. Note: GFP⁺ cells of control rats do not exit sub-epicardial graft site. (B-C') All rats (5/5) that received CSCs primed with CTGF-D4 (3 ng/ml)/Insulin (30 ng/ml) demonstrated robust engraftment of GFP⁺ cells at 1 week after MI. (B, B') Sub-epicardial engraftment (B) and extensive migration/integration (B') of GFP⁺ cells 1 week after MI in a representative animal that received CSCs primed with CTGF-D4/Insulin. (C, C') Engraftment of GFP⁺ cells 1 week after MI in a second representative animal that received CSCs primed with CTGF-D4/Insulin. Image demonstrates integration into area with infarction. FITC channel (C') shows extent of migration into infarct. Note: White dashes in B' and C' indicate infarct border. Area beyond dashes has few viable myocytes that remain from host. Images A, B, B', C, C': Scale bars = 50 μ m; image A': Scale bar = 100 μ m.

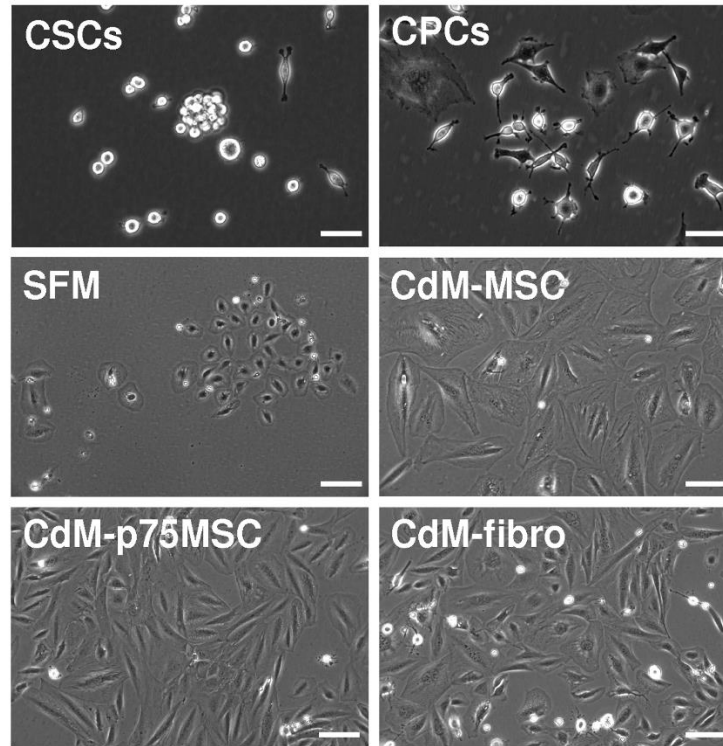


Figure 2- S 1. CSC/CPC culture *ex vivo*

Top 2 panels: Phase contrast images of CSCs, CPCs in CPC growth medium. Lower 4 panels: CPCs treated with CdM from MSCs, p75MSCs, or fibroblasts or SFM for 8 days (magnification, 10x). Please note that only the cells in the lower 4 panels were seeded at the same time and at the same density. CPC growth medium = CSC medium with 2% FBS. Scale bars = 50 μ m.

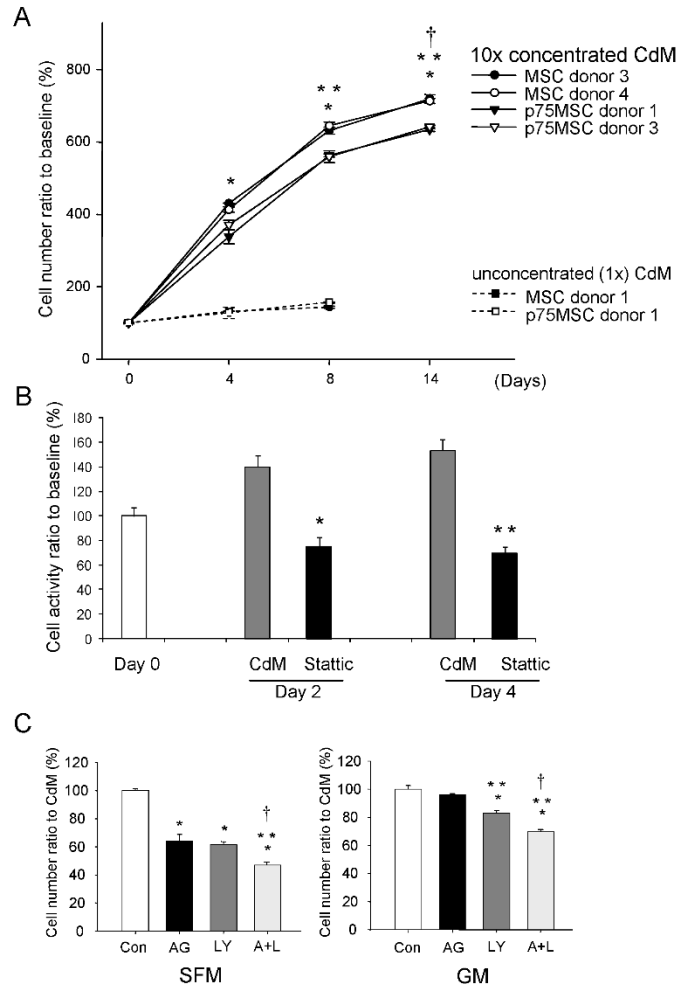


Figure 2- S 2. Proliferation of CPCs

(A) Dose-dependent effect of 10x-concentrated CdM on CPC proliferation. CPC growth in 1x CdM from one MSC donor and one p75MSC donor is shown for reference. Control cell number (60,191 cells) was regarded as 100%. Data are mean \pm SEM, $n = 3$. 10x CdM from 2 different donors was assayed for each cell type. *, $P < 0.0001$ vs baseline; **, $P < 0.0001$ vs day 4; †, $P < 0.05$ vs day 8. CdM, conditioned medium. (B) Growth of CPCs in p75MSC CdM is dependent on signaling through STAT3 and abolished by incubation with the specific STAT3 inhibitor, “Stattic” (10 μ M). *, $P < 0.001$ vs DMSO vehicle on day 2; **, $P < 0.001$ vs. DMSO vehicle on day 4. (C) Inhibitory effects of AG490 (10 μ M) and LY294002 (10 μ M) on CPCs incubated with 1x CdM for 48 hrs. Control cell numbers (99,965 cells in SFM, and 164,614 cells in GM) were regarded as 100%. For C, data are mean \pm SEM, $n = 3$ to 6. *, $P < 0.0001$ vs control; **, $P < 0.01$ vs AG; †, $P < 0.05$ vs LY. Con: control, DMSO. AG: AG490, Jak2/STAT3 pathway inhibitor. LY: LY294002, inhibitor of PI3K/Akt pathway. A+L: AG490 + LY294002. CdM, conditioned medium. SFM, serum-free α -MEM (Left). GM, CPC growth medium (CSC medium with 2% FBS) (Right).

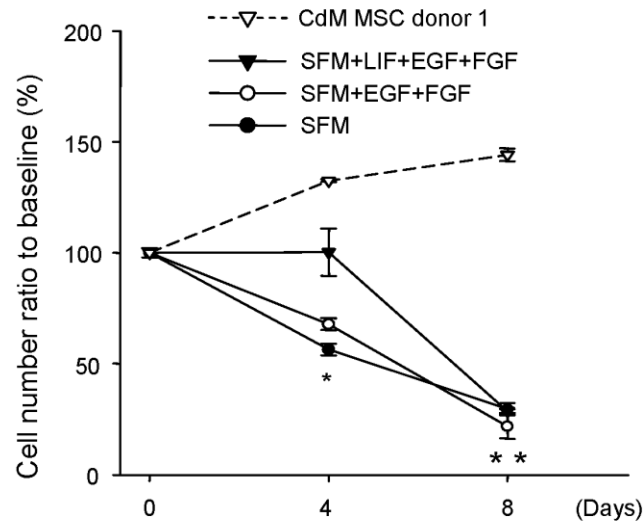


Figure 2- S 3. Growth factor administration on CPCs

Time course changes in the numbers of CPCs treated with SFM supplemented with various growth factors (EGF, bFGF, and LIF; 10 ng/ml) and in the absence of Insulin-Transferrin-Selenium. Control cell number (64,026 cells) was regarded as 100%. Data are mean \pm SEM, $n = 3$. *, $P < 0.01$ SFM and SFM+EGF+FGF vs baseline; **, $P < 0.001$ SFM, SFM+EGF+FGF, and SFM+LIF+EGF+FGF vs baseline. Data for growth in 1x CdM from one MSC donor is shown for reference.

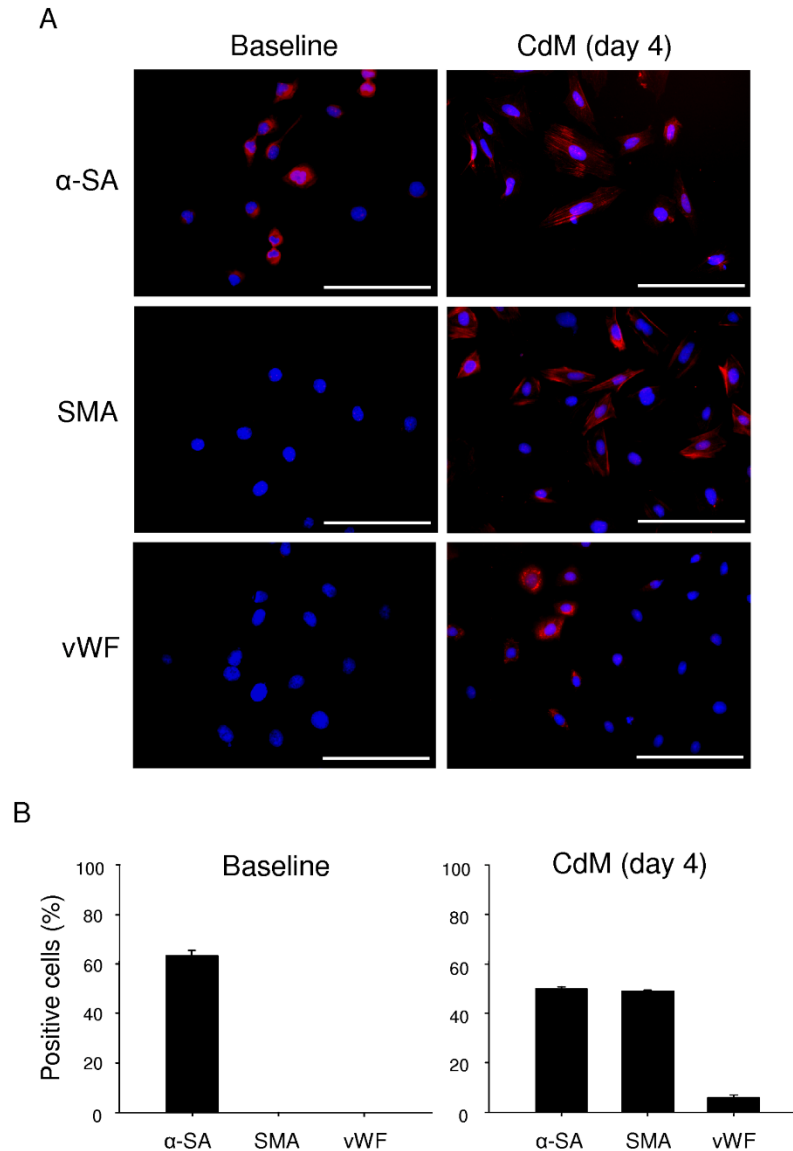


Figure 2- S 4. Differentiation of CPCs expanded in CdM

(A) Immunofluorescent staining for α -SA, α -sarcomeric actin; SMA, α -smooth muscle actin; and vWF, von Willebrand factor. Left panels (Baseline) show the CPCs in growth medium 3 days after plating, and the right panels show CPCs expanded in CdM for 4 days. (B) Quantification of % positive cells for α -SA, SMA, and vWF at for cells incubated in growth medium, CdM and CTGF-D4 (3 ng/ml)/Insulin (30 ng/ml). Data are mean \pm SEM, n = 3. CdM, conditioned medium, Scale bars = 100 μ m. Note; the total count for 3 lineages exceeds a 100% as a portion of CPC-derivatives in culture stain positive for both SMA and α -SA after 4 days of differentiation under the above conditions.

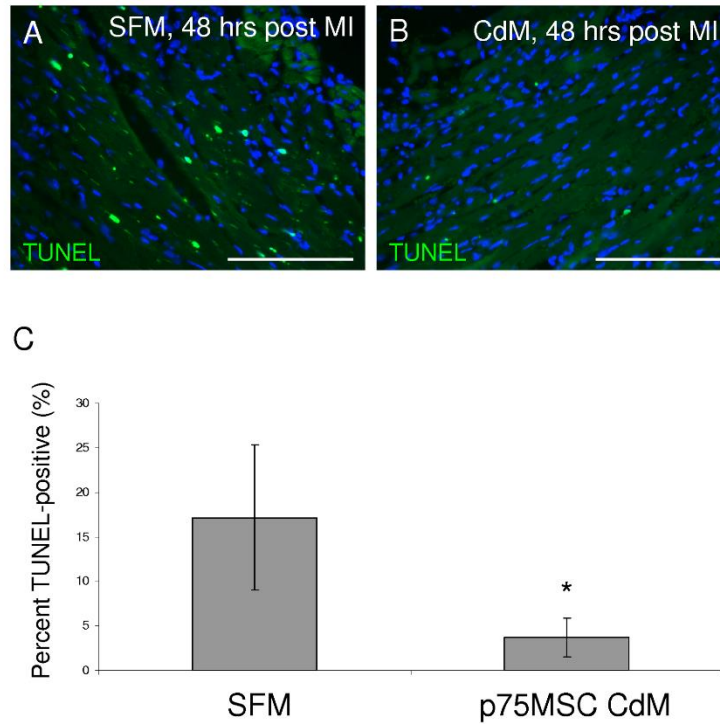


Figure 2- S 5. p75CdM *in vivo* administration

Intra-arterial infusion of p75MSC CdM 24 hrs after MI significantly reduces the level of cardiac apoptosis/necrosis at 48 hrs after MI in adult mice. (A) TUNEL stains of heart sections from vehicle (SFM-treated) and CdM-treated C57bl6 mice. At 24 hrs after LAD ligation, 200 μ l of SFM or 30x p75MSC CdM was slowly infused into the left ventricle lumen (intra-arterial delivery). (B) Quantification of TUNEL⁺ cells in heart sections of animals that received intra-arterial infusion of SFM or 30x p75MSC CdM 24 hrs after MI. Scale bars = 100 μ m.

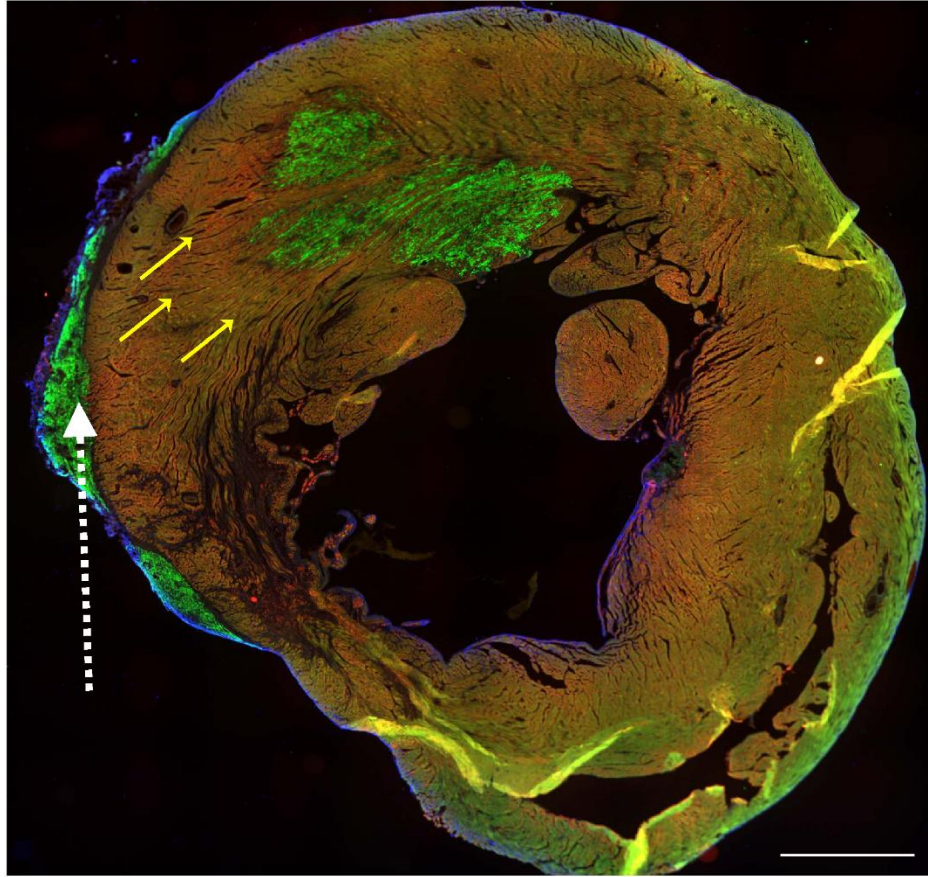


Figure 2- S 6. Coronal section after engraftment

CSCs primed with 30x p75MSC CdM engraft into sub-epicardial tissues after MI and migrate into specific zones with infarction. Image shows the localization of CSC-derived GFP⁺ cells, 1 week after MI and CSC/CdM injections. The dashed white arrow indicates the direction of the tangential sub-epicardial injection. The small yellow arrows indicate the direction of cell migration into the myocardium from the sub-epicardial space. Scale bars = 50 μ m.

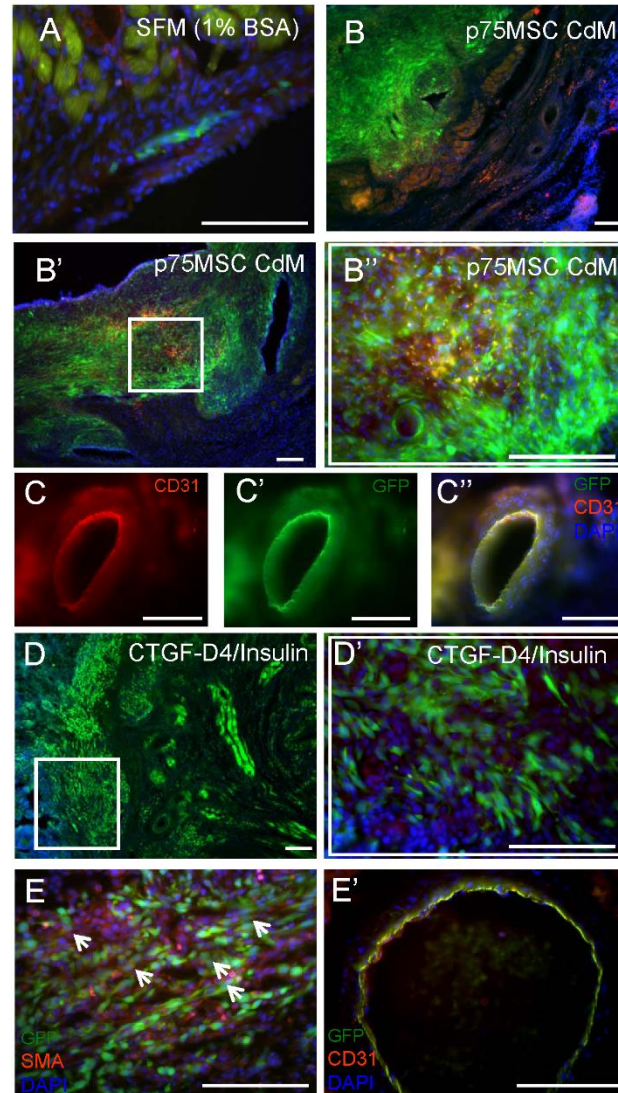


Figure 2- S 7. One month engraftment data

CSCs primed with 30x p75MSC CdM survive and differentiate in the heart 1 month following MI and transplantation. (A) Few cells survive after vehicle control injection (1% BSA in alpha MEM) 1 month post MI. (B, B') Two different representative animals grafted with GFP-positive CSCs. Cells were primed with 30x p75MSC CdM and populate the majority of the infarct after 1 month. (B'') High magnification view, highlighting GFP-positive cells in the core of an infarct. (C, C', C'') Engrafted CSCs differentiated into CD31-positive endothelial cells and contributed to large blood vessels. (D, D') CSCs and their derivatives, engrafted with CTGF-D4/Insulin, 1 month after injury. (E-E') Engrafted CSCs differentiated into smooth muscle actin (SMA)-positive myofibroblasts (E) and CD31-positive endothelial cells (E') and also contributed to large cardiac blood vessels. Scale bars = 100 μ m.

**CHAPTER III. CTGF-D4 ENGAGES AN LRP6/SOX9/ETB_R
SIGNALING AXIS THAT PROMOTES THE EPICARDIAL
RESPONSE TO INJURY AND GRAFT SUCCESS AFTER
MYOCARDIAL INFARCTION**

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Abstract

Transplantation of stem/progenitor cells holds promise for cardiac regeneration in patients with myocardial infarction (MI). However, low cell engraftment continues to present a major challenge and ligands, receptors, and signaling pathways that promote graft success remain poorly understood. Here we prospectively isolate uncommitted epicardial cells from the heart cover by CD104 (beta-4 integrin) and demonstrate that C-terminal peptide from Connective Tissue Growth Factor (CTGF-D4), when combined with insulin, effectively primes adult epicardial derivatives for robust cardiac engraftment after MI. By neutralization and knockdown studies, we determined that CTGF-D4 signaled through Wnt co-receptor LRP6, triggering Sox9-dependent expression of Endothelin Receptor B (ETB_R) and epicardial cell migration. In loss-of-function studies, conditional deletion of ETB_R in Keratin18-expressing epicardial cells prevented their proliferation, EMT, and migration into myocardium after MI. Our results illustrate an LRP6/Sox9/ETB_R signaling axis that controls the biology of native epicardial cells and derivatives and epicardial graft success after MI.

Introduction

The limited endogenous regenerative capacity of the adult mammalian heart has prompted substantial investment and effort into cell-based therapy for acute myocardial infarction (MI) (Laflamme and Murry, 2011; Wadugu and Kuhn, 2012). The ability to provide robust, persistent cell grafts in a predictable manner is highly desirable and may dramatically improve cardiac regeneration. To date, however, despite numerous animal studies and human trials aimed at treating MI and heart failure through direct cardiac cell replacement, clinical use of many promising cell types remains hampered by low levels of cell survival, engraftment, and differentiation following transplantation.

Epicardial cells and derivatives from the heart cover are receiving increasing attention as a promising cell source for cardiac repair after injury (Bollini et al., 2014; Braitsch et al., 2013; Gittenberger-de Groot et al., 2012; Gittenberger-de Groot et al., 2010; Gonzalez-Rosa et al., 2012; Riley, 2012; Ruiz-Villalba et al., 2015; Singh and Epstein, 2012; Smart et al., 2013). Adult epicardial cells are multi-potent and undergo proliferation and epithelial to mesenchymal transformation (EMT) after MI; this results in subepicardial thickening adjacent to areas of injury (e.g. infarct border zones) (Gonzalez-Rosa et al., 2012; Smart et al., 2013). From there, epicardial-derived cardiac precursor cells migrate into injured myocardium and participate in myocardial repair and remodeling (Duan et al., 2012; Ren et al., 2013; van Wijk et al., 2012; Zhou and Pu, 2011). Multiple signaling factors such as Retinoic Acid, FGF and Wnt have been shown to regulate the activities of epicardial cells during development (Rentschler and Epstein, 2011; Vega-Hernandez et al., 2011; Zamora et al., 2007). In contrast, for adults with cardiac injury, extrinsic factors

that regulate epicardial cell behavior and/or functions are poorly understood and none that mediate epicardial engraftment have been identified.

Recently, we reported a new biologic grafting drug based on a defined combination of human Connective Tissue Growth Factor (C terminal, 4th domain peptide; CTGF-D4) and insulin (Iso et al., 2014). This peptide combination effectively primes cultured cardiac progenitor cells to graft into subepicardial sites bordering tissue with infarction after MI (Iso et al., 2014). Here we identify Wnt-co receptor LRP6 as a key target for CTGF-D4 that mediates epicardial graft success and define an LRP6/Sox9/ETB_R signaling axis that controls multiple aspects of the epicardial response to injury. Through loss-of-function studies using inducible, conditional knockout (cKO) mice with an epicardial cell-specific promoter (Keratin18CreERT²-ETB_R-cKO mice), we demonstrate that ETB_R is required for epicardial proliferation, EMT and migration into the myocardium after MI. Our results illustrate overlap between the signals and signaling pathways that regulate injury-induced responses of native epicardial cells and those that promote grafting of culture-expanded cells to subepicardial sites after MI.

Results

CD104 is a specific marker of undifferentiated adult epicardial cells with an epithelial-like (mesothelial) phenotype

There is growing interest in use of epicardial cells and their derivatives for regenerative medicine (van Berlo and Molkentin, 2014; Vieira and Riley, 2011), but lack of defined cell surface epitopes unique to adult epicardial cells makes their isolation and enrichment difficult. To genetically-label and study epicardial cells *in vivo*, several studies have utilized gene expression of epicardial cell-associated transcription factors such as Wt1

(Zhou et al., 2008), Gata5 (Merki et al., 2005), Tbx18 (Cai et al., 2008) and Tcf21 (Acharya et al., 2011). While this approach is useful, it is less effective for clinical applications due to the requirement for genetic manipulation of the organism and overlapping expression of these transcription factors in downstream epicardial derivatives and non-epicardial cells (Ali et al., 2014; Kikuchi et al., 2011; Wagner et al., 2008). To effectively harness epicardial cells and their derivatives for clinical use, standardized protocols for cell isolation and preparation that are less invasive will be necessary. Accordingly, we sought to develop methods for direct epicardial cell isolation based on differential expression of cell surface epitopes.

To distinguish them from other cardiac cell types, we focused on the epithelial-like phenotype of adult epicardial cells and determined that beta 4 integrin (CD104) alone could be used to enrich specifically for adult epicardial cells. A transmembrane glycoprotein, CD104 forms a heterodimer with alpha 6 integrin, thereby modulating epithelial cell proliferation and adhesion to the basement membrane (Kajiji et al., 1989). Several reports used CD104 to identify epithelial stem cells from various organs/tissues including lung, dental pulp, urothelium and eye (Chapman et al., 2011; Kurzrock et al., 2008; McQualter et al., 2010; Pajooresh-Ganji et al., 2006). By immunohistochemistry, we found that CD104 was expressed specifically by the epicardium in tissue sections from rat, mouse and man (Figures 1A). Furthermore, Magnetic-Activated Cell Sorting (MACS) using an antibody to CD104 selectively purified epicardial cells away from myocytes, fibroblasts, endothelial and hematopoietic cells, as well as downstream (post-EMT) epicardial derivatives (Figure 1B). CD104-isolated cells were positive for epithelial keratins (intermediate filament proteins) and transcription factors such as

Gata4, Wt1, Tbx18, and Tcf21, confirming their epithelial and epicardial identities, respectively (Figure 1C-D) (Bochmann et al., 2010). A subset of Wt1-positive cells also expressed p63, a marker of epithelial cell activation and proliferation (Figure S1B) (McKeon, 2004; Senoo et al., 2007).

During 3-7 days of culture in medium containing 10% FCS, epicardial cells isolated by cell surface CD104 underwent epithelial to mesenchymal transformation (EMT) (Figure S1A). Epicardial EMT was confirmed by the appearance of CD90 (Thy-1), a cell adhesion protein expressed during differentiation (Lennon et al., 1978; Bakondi et al., 2009) (Figure 1E), and concomitant loss of CD104 (Figure 1F). Hereafter, epicardial cells expressing Vimentin (mesenchymal intermediate filament protein, Figure 1G) and CD90 (Figure 1H) after EMT are referred to as Epicardial-Derived Cells (EPDC).

CTGF-D4 promotes primary EPDC grafts to adult hearts with MI

Detected by *in situ* hybridization at 1 week after MI, CTGF mRNA expression was reported to increase markedly in subepicardial fibroblasts bordering tissue with infarction (Chuva de Sousa Lopes et al., 2004). In agreement, by immunohistochemistry with antisera specific to CTGF-D4, we localized CTGF-D4 at 1 week after MI to border zone subepicardial regions in hearts from both rats and mice (Figure 2A). In a recently published neutralization screen, we identified CTGF and insulin as factors present in medium conditioned by human bone marrow progenitor cells that acted synergistically to increase the survival of cardiac progenitor cells (CPCs) during simulated ischemia (1% oxygen with nutrient deprivation). Furthermore, using a permanent ligation model of MI,

we determined that cultured CPCs could be primed with a combination of CTGF-D4 and insulin to promote robust graft success with CPCs transplanted into subepicardial sites bordering tissue with infarction (Iso et al., 2014). Although our screen identified insulin independently, a previous report had shown that insulin-like growth factor (IGF-1) could enhance engraftment and differentiation of murine ES cells when co-injected into hearts with myocardial infarction.

The endogenous subepicardial expression pattern of CTGF-D4 and its ability to foster cell grafts at subepicardial sites prompted us to investigate the effect(s) of CTGF-D4 on primary adult epicardial cells. In culture, CTGF-D4 promoted EPDC adhesion to fibronectin-coated glass cover slides (Figure S2), suggesting that it might support engraftment of transplanted EPDCs. To mimic a clinical scenario in which patients are revascularized after acute MI, we used a different MI model with a period of myocardial ischemia followed by reperfusion (MI/R) (Rao et al., 2015). To test the ability of CTGF-D4/Ins to prime EPDC, we performed MI/R surgeries in adult rats (2 h ischemia followed by reperfusion), and injected DiI-labeled EPDC into 2 subepicardial sites at the time of reperfusion. We evaluated graft success at 1 week or 1 month after cell injection. In contrast to vehicle-primed cells, EPDC primed by CTGF-D4/Ins effectively grafted the subepicardium in regions bordering the infarct, proliferated (see Ki67⁺ EPDCs in Figure S3B), and migrated into the injured myocardium (Figure 2B). At 1 week after MI/R and cell injection, cell counts from selected serial cardiac tissue sections containing the highest number of DiI-labeled cells (1 slide per heart) demonstrated a significant increase in EPDC engraftment for animals injected with CTGF-D4/Ins-primed EPDC as opposed to vehicle-primed EPDC (vehicle, 350 ± 49 cells; CTGF-D4/Ins, $14,868 \pm 111$ cells; $p \leq$

0.001, n=6 per group; Figure 2C, Figure S3A). Since DiI-labeling does not persist long-term after cell proliferation, we repeated the MI/R study with male to female grafts and quantified the level of male EPDC engraftment using real-time quantitative PCR (qPCR) assays for Y chromosome. After 1 month, we detected significantly more male DNA in female hearts that received CTGF-D4/Ins-primed EPDC compared with injections of vehicle-primed EPDC (vehicle, $.0031 \pm .00119\%$; CTGF-D4/Ins, $0.41367 \pm .0244\%$; $p \leq 0.001$, n=4 per group; Figure 2D).

By echocardiography (ECHO), hemodynamic measurements 1 week after MI/R and cell transplantation were similar between the two groups (Figure S3C). However, after 1 month we observed a significant improvement in cardiac function for animals grafted with CTGF-D4/Ins-primed cells. LV fractional shortening (FS), ejection fraction (EF) and anterior wall thickness (AWT) were greater in systole (S) and diastole (D) in animals treated with CTGF-D4/Ins-primed cells compared with controls (% FS; sham, $38.65\% \pm 3\%$, vehicle, $30.34 \pm 3.4\%$; CTGF-D4/Ins, $40.81 \pm 7.8\%$; vehicle vs. CTGF-D4/Ins, $p \leq 0.05$; % EF; sham, $73.0\% \pm 7.2\%$, vehicle, $59.62 \pm 6.1\%$, CTGF-D4/Ins, $71.91 \pm 5.33\%$; vehicle vs. CTGF-D4/Ins, $p \leq 0.05$; AWT (S-D), sham, $0.29 \pm .043$, vehicle, $0.19 \pm .03$, CTGF-D4/Ins $.274 \pm .031$; vehicle vs. CTGF-D4/Ins, $p \leq 0.05$; Figures 2E-G).

Identification of LRP6 as a receptor for CTGF-D4 on epicardial cells

CTGF-D4 has been shown to interact with integrins, and multiple cell surface receptors including TrkA, EGFR, FGFR2, LRP1 and LRP6 (Figure 3A) (Aoyama et al., 2012; Kawata et al., 2012; Mercurio et al., 2004; Ren et al., 2013; Wahab et al., 2005) .

Exposure of EPDC to CTGF-D4 led to increased expression of CD90 and other differentiation markers such as alpha smooth muscle actin (α -SMA, smooth muscle cells and myofibroblasts), and von Willebrand Factor (vWF, endothelial cells) (Figure 3B). Notably, prior incubation in neutralizing antibodies against LRP6 reduced the differentiating effects of CTGF-D4 (Figure 3B).

We next examined whether CTGF-D4/LRP signaling affected EPDC survival. Under conditions of simulated ischemia, blocking antibodies directed to both Wnt co-receptors (LRP5/6) diminished the protective effects of CTGF-D4. Notably, blocking LRP6 alone had the same effect (Figure 3C). Furthermore, binding LRP6 to its ligand Dkk-1 abolished CTGF-D4 mediated EPDC protection, showing that LRP6 regulates CTGF-D4-dependent cell survival (Figure S4). Altogether, these results demonstrate a role for LRP6 in relaying signals from extracellular CTGF-D4 during EPDC differentiation and survival.

LRP6 signaling is required for EPDC engraftment following transplantation

To test the hypothesis that CTGF-D4 signals through LRP6 to promote EPDC graft success after myocardial infarction, we developed a competitive dual engraftment assay (Figure 2D, Methods). At 1 week Following MI/R and cell engraftment, we observed a significant reduction in the grafting efficiency of EPDC when blocked with anti-LRP6. To further control for the process of dye labeling we performed reciprocal dye labeling of EPDC and Fc fragmentation of the blocking antibody prior to engraftment. Importantly, we performed the Fc fragmentation step to ensure that the Fc segment of the blocking antibody did not activate immune cells and preferentially eliminate epicardial cells that

were bound to antibody (see Supplementary methods) (Cell number from best tissue section: Control IgG, 29.871 ± 2301 cells; anti-LRP6 3934 ± 100 cells; $p \leq 0.01$) (Figure 3E-E',F). We next carried out male cell transplants to female donors to confirm results from the competitive engraftment assay at 1 month after MI. Assays of genomic DNA isolated from whole LV revealed a significant decrease in EPDC engraftment when LRP6 was blocked, compared with cells incubated in control IgG (Figure 3G).

CTGF-D4/LRP6/Sox9 signaling targets ETB_R to control epicardial cell migration

To identify other cell surface receptors and pathways that potentially interacted with CTGF-D4 signaling and its effects on epicardial cells, we performed an inhibitor screen with medium containing CTGF-D4 and normoxic (21% oxygen) or hypoxic (1% oxygen) conditions. The results showed that inhibition of either FGFR/VEGFR or Endothelin Receptor Type B (ETB_R) reduced epicardial cell numbers (Figure S5). In contrast, inhibitors for EGFR, PDGFR, MAPK, Notch signaling (Gamma-secretase) and Endothelin Receptor Type A (ETA_R) did not (Figure S5). As role(s) for FGFR and VEGFR had been established for epicardial cells (Lepilina et al., 2006; Vega-Hernandez et al., 2011), we chose to focus on ETB_R.

By antibody staining, ETB_R was expressed by native epicardial cells as they migrated into the myocardium after MI (Figure 4A). For EPDC in culture, CTGF-D4 treatment increased ETB_R, whereas blocking LRP6 with a neutralizing antibody abolished the effects of CTGF-D4 (Figure 4B-C). Furthermore, in a cell migration assay (Figure 4D), we found that an ETB_R agonist (IRL-1620) promoted EPDC migration, while,

antagonizing ETB_R blocked EPDC migration (Figure 4E-F); these results indicated that ETB_R signaling could be an important determinant of epicardial cell migration.

We next sought to identify intracellular factor(s) through which CTGF-D4/LRP6 signaling could act to promote ETB_R expression. Previous reports had demonstrated Sox9, a transcription factor, was important for epicardial EMT during development and after injury (Hofsteen et al., 2013; Smith et al., 2011). In addition, Sox9 was found to control the EMT process in adult cancers of the breast, skin and lung (Capaccione et al., 2014; Cheng et al., 2015; Guo et al., 2012). Relevant to our studies, CTGF was shown to induce epithelial cell hyperplasia in the lung by increasing Sox9 levels (Sonnylal et al., 2013). Accordingly, we examined whether primary isolates of EPDC expressed Sox9, and they did (Figure 4G). Subsequent treatment of EPDC with CTGF-D4 demonstrated increased Sox9 levels, an effect that was diminished by blocking LRP6 prior to CTGF-D4 treatment (Figure 4H-I).

Having observed similar changes in Sox9 and ETB_R expression after CTGF-D4/LRP6 stimulation in primary EPDC, we hypothesized that Sox9 controls the level of ETB_R in response to CTGF-D4/LRP6 signaling. To establish the CTGF-D4/LRP6/Sox9 signaling axis, we used lentiviral shRNA to knockdown Sox9 in HEK293 cells.

Coincident with decreased Sox9 levels by shRNA, we observed a corresponding decrease in ETB_R compared with ETB_R levels in cells that received scrambled lentiviral shRNA (control) (Figure 5A). By promoter mapping, we determined the *EDNRB* gene promoter contained several Sox9 binding elements (Figure 5B,C). To demonstrate transcriptional regulation of *EDNRB* by Sox9, we transfected control HEK293 cells (scrambled shRNA) and Sox9 knockdown HEK293 cells with a pGL4 luciferase reporter construct containing

EDNRB promoter DNA (−3,022 bp to +160 bp of the human promoter) (Yokoyama et al., 2006) (Figure 5D). Following treatment with CTGF-D4, *EDNRB* promoter activity increased in the control cells, but not in the Sox9 knockdown cells (Figure 5E). Collectively, these results suggest that CTGF-D4 signals through LRP6 and Sox9 to control the levels of ETB_R.

ETB_R controls epicardial cell proliferation after MI

To follow adult epicardial cells and their derivatives *in vivo*, we produced a lineage-tracing strain using transgenic Rosa26-mTmG reporter mice, in which a stop codon flanked by loxP recombination sites lies between a constitutively-expressed gene for a red fluorescent reporter (tdTomato) and an inducible gene that encodes a green fluorescent reporter (GFP) (Muzumdar et al., 2007). For cell labeling, the Rosa26-mTmG strain was mated to mice that expressed a Tamoxifen (TM)-inducible form of Cre recombinase under control of the Keratin18 (K18) promoter, which distinctly labels epithelial cell populations throughout the body (Figure S6A) (Ousset et al., 2012; Van Keymeulen et al., 2009; Van Keymeulen et al., 2011). In the heart, Keratin18 is expressed exclusively by epicardial cells; this is true for multiple species from development through adulthood (Figure 6A) (Chen et al., 2002; Eid et al., 1992; Schaefer et al., 2004; Thorey et al., 1993; Vrancken Peeters et al., 1995). In confirmation of the model, treatment of 1week atrial explant cultures from K18CreER^{T2}-mTmG mice with 4-hydroxy-Tamoxifen resulted in epicardial cell-specific recombination (Figure 6B). Using the K18CreER^{T2}-mTmG mice, we specifically labeled epicardial cells on the heart surface (i.e. epithelial phenotype) and then monitored epicardial cell proliferation,

epicardial EMT, and EPDC migration after MI (Figures 5B). Tamoxifen (TM) injections initiated recombination in $41 \pm 3.2\%$ of epicardial cells on the heart surface in healthy control mice and in $46.01 \pm 4.89\%$ of cells for mice with MI (Figure 6C). As expected, at 3 days after MI we observed Ki67⁺ proliferating cells in both subepicardial and myocardial regions adjacent to and within the infarct (Figure 6D,D'). In confirmation of our inducible transgenic mouse model, at 3 days after MI we observed K18-positive epicardial cells that underwent EMT and GFP⁺ EPDC that subsequently migrated into border zone subepicardial sites (Figures 6E).

To determine whether ETB_R had specific role(s) in the proliferation, migration and/or function of adult epicardial cells and their derivatives, we generated K18-CreER^{T2}-ETB_R-cKO mice (Figure S6C-D). To measure the effects of ETB_R cKO after MI/R, we used double immunohistochemistry to develop a simple K18-based cell quantification index for both the infarct core and adjacent border zone areas of K18-CreER^{T2}-ETB_R-cKO mice. Importantly, we found that K18 staining persisted in murine epicardial-derived CD90⁺ cells after EMT and thus could be used, in the short-term, to track epicardial cells and EPDC after MI/R. At 3 days after TM administration and MI/R surgery, we performed blinded counts to ascertain the number of K18⁺/Ki67⁺ proliferating epicardial cells and the number of K18⁺/CD90⁺ epicardial cells that had undergone EMT in the presence/absence of ETB_R cKO. As expected, after MI/R we observed significant proliferation of subepicardial cells and subepicardial thickening in vehicle (oil)-treated animals (Figures 6F-G). Surprisingly, however, K18⁺ epicardial cells in TM-treated animals did not proliferate, nor did they generate a thickened subepicardial cell layer (Figures 5F-G). For TM-treated mice, the number of K18⁺/Ki67⁺ epicardial

cells in border zone regions was significantly less than that of vehicle-treated mice (Oil, $43.55 \pm 16.48\%$; TM, $13.35 \pm 6.65\%$; $n=5$ per group, $p \leq 0.01$)(Figure 6F).

To evaluate epicardial-specific EMT we analyzed 2 distinct populations of epicardial cells at 3 days after MI/R: 1) K18⁺ only cells (epicardial cells that did not undergo EMT) and 2) K18⁺/CD90⁺ cells (epicardial-derivatives post-EMT) in regions bordering infarct. As expected, within the infarct zones of vehicle-treated animals we observed that the majority of epicardial cells underwent EMT by 3 days after MI/R. As reported in patients with chronic cardiac failure, these areas were nearly devoid of K18⁺ epicardial cells (Figure 5H). In stark contrast, within the infarct zones of TM-treated K18-CreER^{T2}-ETB_R-cKO mice, we observed a significant percentage of K18⁺ epicardial cells that maintained an epithelial phenotype (CD90-negative). Intriguingly, these cells had failed to undergo EMT and thus now remained on the heart surface (Epicardial K18⁺ cells [infarct zone]: Oil, $11.01 \pm 2.67\%$; TM, $78.45 \pm 9.33\%$; $n=5$ per group, $p \leq 0.01$) (Figures 5H-I). Additionally, within border zone regions, the number of K18⁺/CD90⁺ (double positive) cells in TM-treated animals was significantly less than that of vehicle-treated animals (Subepicardial K18⁺/CD90⁺ cells [border zone]: Oil, $29.99 \pm 4.37\%$; TM, $2.15 \pm 1.98\%$; $n=5$ per group, $p \leq 0.001$) (Figure 5H-I). Notably, for regions distal to the infarct zone and cell necrosis, the number of K18 cells was similar for both the TM- and vehicle-treated groups (Epicardial K18⁺ cells [distal from infarction]: Oil, $62.98 \pm 5.31\%$; TM, $68.12 \pm 6.81\%$; $n=5$ per group) (Figure 5J-K).

Discussion

Here we report multiple effects of CTGF-D4 on epicardial cells that include induction of EMT and promotion of differentiation, migration, and survival. By performing cell engraftment and *ex vivo* cell signaling studies, we report that CTGF-D4 engages a previously unknown LRP6/Sox9/ETB_R signaling axis that appears critical for the epicardial response to MI.

The influence of CTGF in a cardiac injury environment is of great interest and needs further investigation. Whereas endogenous cardiac CTGF expression is low or absent in healthy myocardial tissue and non-infarcted tissue after MI (Dean et al., 2005), it is rapidly expressed in border zone subepicardial tissue early after MI. We found that injection of CTGF-D4/Ins-primed EPDCs into border zone subepicardial tissue as opposed to sites distal to infarction was necessary for successful cell engraftment. This difference in capacity to accept and propagate cellular grafts implies that additional extrinsic factors present within activated subepicardial tissue, perhaps even CTGF, are necessary for graft success.

Our studies do not exclude the possible role for CTGF-D4 in stimulating or mediating other cellular pathways in primary epicardial cells, aside from the signaling axis we report. For instance, during development, in epicardial progenitor cells beta-catenin is required for epicardial EMT and coronary artery formation during development (Zamora et al., 2007). Observations in our lab to detect differences in nuclear localization of beta-catenin or using BATGAL mice to detect Wnt pathway activation by beta-catenin promoter activity in our lab did not indicate that CTGF-D4 acted on LRP6 like a typical

Wnt ligand. Rather, our results in primary adult epicardial cells agree with those of the Tallquist group for beta-catenin in epicardial cells during development (Wu et al. 2010). Here, they showed that the epicardium is a polarized epithelium and that beta-catenin in epicardial adherens junctions and is required for critical epicardial processes including EMT and cell division. These effects were independent of beta-catenin transcriptional activity (Wu et al., 2010). As such, CTGF-D4/LRP6 signaling in adult epicardial cells and EPDC may alter beta-catenin dynamics in a non-canonical fashion, affecting proteins involved with cell polarity, cell division and EMT.

Alternatively or in concert, CTGF-D4/LRP6 signaling may control the movement or function of transcriptional coactivators such as Yes-associated protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ), key components of the Hippo pathway. These proteins form regulatory complexes that interact with TEAD transcription factors to promote EMT (Diepenbruck et al., 2014). Of special interest, CTGF itself is a direct transcriptional target of YAP and TAZ and TEAD (Zhao et al., 2008; Zhang et al., 2009).

Through experiments that combined Sox9 gene knockdown with ETB_R promoter activity assays, we identified Sox9 as a novel transcriptional regulator of ETB_R. Interestingly, for mature cardiac myocytes and smooth muscle cells, endothelin (ET-1) signaling through ETA_R was shown to increase CTGF expression (Rodriguez-Vita et al., 2005). By contrast, our inhibitor screen with CTGF-D4 and adult epicardial derivatives identified FGFR and ETB_R, but not ETA_R. For pancreas development, Sox9 acts as the “centerpiece” in a Sox9/Fgf10/Fgfr2b feed-forward signaling loop that promotes both proliferation and fate-commitment of pancreatic progenitor cells (Seymour et al., 2012).

Given the known role of Fgfr2b signaling in controlling epicardial movement through the myocardium during development (Vega-Hernandez et al., 2011), signaling through CTGF-D4/LRP6 and Sox9 may mediate an increase in Fgfr2b to promote EPDC survival and/or migration after cardiac injury in adults. In this manner, ETB_R and Fgfr2 could act as downstream effectors of CTGF-D4 signaling that participate in co-incident or related functions during epicardial EMT and/or migration.

All these observations with regard to its expression pattern after MI and the numerous potential roles for CTGF-D4, taken together with our observations suggest that exogenous delivery of CTGF-D4 to areas bordering infarction may enhance proliferation and/or migration of native epicardial cells to improve cardiac regeneration and function after MI. Furthermore, because most adult cell types, including stem/progenitor cells, possess the insulin receptor and also receptors and/or integrins that bind CTGF-D4, the strategy of CTGF-D4/Ins-mediated priming and transplantation to subepicardial sites after MI may promote graft success for other reparative cell types such as adult multipotent stromal cells (MSCs), cardiac-specified progenitors (CPCs), or derivatives from embryonic stem cells (ES cells).

Experimental Procedures

Isolation and short term priming of primary EPDC

Rat atrial explants were cultured in 10% fetal bovine serum containing medium for 48 hours, at which point primary epicardial cells were purified from all other cell types by magnetic sorting using anti-CD104 antibody. EPDCs were cultured as adherent cultures

in 10% complete culture medium. Cells were counted by hemocytometer, centrifuged, and re-suspended in CTGF-D4 (40 ng/ml)/Insulin (40 ng/ml)/1% BSA in α -MEM, or 1% BSA in α -MEM (vehicle control for recombinant peptides).

Myocardial infarction and ischemia reperfusion surgery

All animal work was approved by the University of Vermont College of Medicine's Office of Animal Care in accordance with the American Association for Accreditation of Laboratory Animal Care, as described in (Iso et. al. 2013).

Quantitative real-time PCR for Y chromosome

Rat genomic DNA was isolated from the left ventricle homogenates in of rats by extraction with SDS/ Proteinase K digestion and Tris buffered phenol at pH 8.0. DNA yield and purity were verified then utilized as template for quantification of rat Y chromosome by real-time PCR in an automated instrument (ABI Prism 7700 sequence detection system, Applied Biosystems). Taqman PCR master mix was used to perform PCR reactions as per protocol (ABI Perkin Elmer). Y chromosome probe (5'-FAM CAA CAGAATCCCAGCATGCAGAATTCA 3' -TAMRA) at 250 nM concentration was used with the primers (forward 5' GGAGAGAGG CACAAGTTGGC 3', reverse 5' CCCCAGCTGCTTGCTGATC 3'; Integrated DNA Technologies, Coralville, IA) at 900 nM concentrations. Standard curve preparation and analysis were performed as described in previously published work (Spees et. al., 2003)

Migration assay

The setup was adopted from a previously described study (McKenzie et. al., 2011). 3000 Primary EPDC were plated inside the core of a silicone donut, on fibronectin (20µg/ml) coated glass cover slips. After overnight adhesion, nuclei were stained using Hoechst 33342 nuclear stain (Invitrogen, 1:2000 in media) for 15 min at 37°C. The staining media was removed and replaced with either serum free media or serum free media containing Endothelin-1 (20 ng/ml, R&D Systems) with drug vehicle control (DMSO) or pharmacologic agents IRL-1620 (40 µm, Calbiochem) or BQ-788 (8 µm, Calbiochem).

Sox9 shRNA knockdown

Lentiviral transduction with shRNA was used to knockdown Sox9 expression (Mission Lentivirus particles; Sigma Aldrich) in HEK293 epithelial cells. For sequence information and method, refer supplementary methods.

Luciferase assay to measure promoter activity

Human embryonic kidney 293 cells with control scrm shRNA, or Sox9 shRNA transfected with the pGL4:hEDNRB promoter construct in 10% CCM with Lipofectamine 3000 (Life technologies). After 24 hr the cells were seeded into 96-well plates at a density of 10,000 cells per well and allowed to adhere overnight. At 45 hr after transfection, medium was replaced with MEM containing 1% BSA or 1% BSA and CTGF-D4 (40 ng/ml), and CTGF-D4 with control IgG or anti-LRP6 blocking antibody (2 µg/ml). The plate was allowed to incubate further for 3 hours to the point when promoter activity was assessed by luminescence measures with a Synergy HT Multimode Microplate reader (BioTek Instruments, Inc.; Winooski, VT).

Lineage tracing and conditional knockout mice

Transgenic mice expressing Cre recombinase enzyme under the human Keratin 18 promoter were crossed with either mT/mG mice for lineage tracing or to ETB_R flox/flox mice. Refer supplementary methods for genotyping primers and results. Adult male C57BL/6J mice (6–8 wk of age) were obtained from Taconic Farms. Keratin18 Tg mice KRT18-cre/ERT2)23B1pn/J (catalog no. 017948), B6; 129-Ednrbtm1Nat/J (EDNRB-flox mice; catalog no. 011080), and mT/mG mice *Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo/J* (catalog no. 0007576) were from JAX.

Quantification of conditional knockout analysis

For each animal, 9 sections were analyzed, maintaining the same section position as accurately as possible across all animals. In each section, the epicardial region in 6 border zone regions to infarct, 3 infarct regions and 3 distal regions of right ventricle were captured as high magnification fluorescent images. A blinded operator then quantified the different cell populations in randomized, coded images.

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Supplementary Material

Isolation, culture, and characterization of primary adult epicardial cells and EPDC

Right and left atrial appendages were dissected from hearts of adult rats (10 to 12 weeks of age) and rinsed three times in sterile PBS containing 100 units/ml penicillin and 100 µg/ml streptomycin. The atrial tissues were minced into 1 mm³ pieces and allowed to adhere for 48 hr in 10% complete culture medium (10% CCM) that consisted of α -MEM (Gibco) supplemented with 10% fetal bovine serum (FBS lot selected for rapid growth of human multipotent stromal cells; Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Mediatech Inc., Hendron, VA). After 48 hours, the explant pieces were lifted (0.25% Trypsin, 2.2 mM EDTA; Mediatech Inc.), and trypsin was deactivated with 10% CCM. The resulting cell suspension was centrifuged at 800 x g for 6 min and then resuspended in 1 ml of Magnetic-Activated Cell Sorting (MACS) buffer (α -MEM containing 1% w/v BSA and 2 mM EDTA). To isolate CD104⁺ adult epicardial cells (epithelial), we added anti-CD104 antibody (1:500, rabbit polyclonal; Bioss) for 30 min at 4°C and mixed cells every 5 min to keep them as a suspension. This was followed by one wash in 25 ml of MACS buffer, and incubation in 2 ml of anti-rabbit magnetic microbeads (1:1000) for 10 min at RT. Excess microbeads were removing by washing with 25 ml of MACS buffer and centrifugation at 800 x g for 6 min. The input bead-conjugated cell population was resuspended in 5 ml MACS buffer and filtered through a 40 µm cell strainer to obtain a single cell suspension. The cell suspension was then passed through a magnetic column (LS-column, Miltenyi Biotech) that had been pre-equilibrated with 5 ml of MACS buffer. The column was washed three times and removed from the magnet. The CD104-positive

fraction was eluted in 5 ml of MACS buffer, centrifuged at 800 x g for 6 min, resuspended in 10% CCM, and plated for characterization by staining, cell culture treatment studies, or culture expansion for EMT and subsequent engraftment *in vivo*.

Characterization of Cell Surface Epitopes

Pellets containing 0.5 to 1 x 10⁶ cells were suspended in 0.5 ml of PBS and incubated for 30 min at 4°C with pre-titered antibodies for flow cytometry: FITC-conjugated anti-CD90 (BD Biosciences Pharmingen, San Diego, CA) or PE.Cy5-conjugated anti-CD104 (Bioss, Germany). After labeling, cells were washed twice with PBS and analyzed by closed-stream flow cytometry (LSR II, Becton Dickinson, Franklin Lakes, NJ).

RNA isolation and RT-PCR

Total RNA was isolated from cell pellets with a commercial kit (RNAqueous, Ambion, Austin, TX). To avoid the possibility of contaminating DNA, total RNA samples were treated with DNase prior to reverse transcription (TURBO DNase, Ambion). Reverse transcription was performed with Superscript III (Invitrogen) in the presence of RNase inhibitor (RNaseOUT, Invitrogen). PCR was carried out with an Eppendorf Master Cycler EP thermal cycler. Target sequences were denatured at 94°C (2 min) followed by 30 amplification cycles of 94°C (30 s), anneal temp (30 s), and 72°C (45 s). The last PCR step extended the products at 72°C for 2 min. RT-PCR products were analyzed on 1% agarose gels.

Myocardial infarction and ischemia-reperfusion surgery

Adult Fischer 488 rats (males, 7 weeks of age) or transgenic mice (7-8 weeks of age) were weighed, shaved, anesthetized under 4% isoflurane, and endotracheally-intubated. Rats were ventilated at a respiration rate of 65 beats per min under a peak inspiration

pressure of 15 cm H₂O (Kent Scientific). Body temperature was maintained at 37°C with a heating pad (Gaymar). Through a dermal incision, a blunt dissection of the fascia was performed and the intercostal muscles were separated. The heart was exposed by retraction of the pericardium to expose the left anterior descending coronary artery (LAD). The LAD was occluded with a 6-0 nylon suture (or 8-0 nylon suture for mice) and occlusion was confirmed by blanching of the anterior free wall of the LV. Animals were allowed to recover off the ventilator.

Reperfusion in rats was performed 3 hours after ligation. Animals were re-intubated and the chest wall was reopened to expose the occluded LAD. With a fine pair of scissors, the suture was cut to release the ligation and restore blood flow through the vessel. Experimental reperfusion was confirmed by the re-pinking of LV tissue. Cell transplantation was performed at the time of reperfusion.

EPDC transplantation

After 24 hours, rats were re-intubated, ventilated, and the chest wall was re-opened. Hearts were exposed to reveal the border zones of the infarct. For each rat, we performed 2 subepicardial injections of 125,000 EPDC (5 µl each, one per border zone) with a 30 gauge Hamilton syringe. The needle was introduced tangentially to the wall of the LV with the bevel facing upward. The syringe was advanced only as far as the bevel edge to access the subepicardial surface of the heart and so as not target the underlying myocardium of the LV. After the injections, the chest wall was closed and rats recovered for 7 or 30 days prior to euthanization.

Competitive dual engraftment

To perform *in vivo* neutralization and engraftment experiments, Fab fragments of LRP6 and rabbit polyclonal IgG antibody were cleaved and purified away from Fc fragments using a Fab fragmentation kit (Thermo Scientific). This step was performed to ensure that the Fc segment of the blocking antibody did not activate immune cells to preferentially eliminate epicardial cells that were bound to antibody. The neutralizing efficiency of the cleaved antibody was confirmed by comparing its ability to block CTGF-D4 mediated cell protection under simulated ischemia. Purified CD104 cells after EMT were counted and split into 2 populations. One of them was labeled with CMFDA (green dye, 50 $\mu\text{g/ml}$; Invitrogen) and incubated with Fab fragments of control IgG (2 $\mu\text{g/ml}$) while the other population was labeled with CMTPX (red dye, 50 $\mu\text{g/ml}$; Invitrogen) and Fab fragments of LRP6 blocking antibody (2 $\mu\text{g/ml}$), for 30 min at 37°C. Cells were then washed thoroughly 3 times in sterile PBS, mixed together in equal numbers and incubated in CTGF-D4 for 30 min on ice. Note: We initially cultured cells from two cell populations together for 72 hr to ensure that the dyes did not intermix and were not cytotoxic to the primary cells. Given the equal number of red and green cells as the input for cell grafting, we could expect 1 of 2 possible outcomes. If LRP6 was an important receptor for the process, blocking LRP6 in the red population would affect their ability to graft, and we would observe a majority of green cells at 1 week after treatment. In the case that LRP6 was not required, blocking it on the red cells would not affect their grafting ability and we would observe a similar number of red and green cells engrafted at 1 week after treatment. To further control for the process of cell staining, we performed control grafts with reciprocal dye-labeling for cells treated with LRP6 blocking antibody and those treated with control (non-specific) IgG.

Echocardiography

Two dimensional, Doppler, and M-Mode echocardiography was performed with a Vevo 770 High-Resolution Imaging System (VisualSonics, Toronto, ON, Canada) by an operator blinded to the treatment groups. Data were recorded from sham-operated rats and rats with MI/R while under isoflurane anesthesia at 7 or 30 days after cell transplantation. All left ventricular dimensions in systole and diastole were measured from M-mode images obtained at the mid-papillary muscle level.

Following ECHO, animals were euthanized and the hearts were removed and rinsed in PBS. For histology, the ventricles were dissected and rinsed in PBS. They were fixed for 2 days in 4% paraformaldehyde in PBS at 4°C. The entire heart was cut as serial sections (20 µm) from apex to base.

Immunocytochemistry/ Immunohistochemistry

Fixed cells or tissue sections were blocked in PBS with 5% normal goat serum containing 0.4% Triton X-100. Prior to antibody staining, for retrieval of certain antigens, some sections were treated with 20 ug/ml Proteinase K. Cells or sections were incubated in a humidified chamber with primary antibody at 4°C overnight, washed three times with PBS for 5 min each and incubated in secondary antibodies at RT for 1 hour. After staining, sections were mounted with DAPI nuclear counterstain (Fluoromount-G with DAPI, Southern Biotech). Images were captured on a Leica deconvolution microscope (Leica DMI6000B) and analyzed using Leica FW4000 software.

Immunoblotting

Cells were homogenized in PBS and lysed in a buffer that consisted of 0.1% sodium dodecyl sulphate (SDS) and complete protease inhibitor cocktail (Roche, Basel,

Switzerland). Protein concentration was determined by the DC protein assay (Biorad, Hercules, CA). Twenty μg of protein was separated by SDS-PAGE. After electrophoresis, the gels were electroblotted to polyvinylidene difluoride (PVDF) membranes. All electrophoresis and electroblotting used Novex reagents and systems (Invitrogen, Carlsbad, CA). The blots were blocked for 1 hr at RT in 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBST), washed three times for 5 min each in PBST, and incubated in primary antibodies in PBST with 5% BSA overnight at 4°C. After three, 5 min washes in PBST, the blots were incubated in secondary antibody conjugated to horseradish peroxidase (1: 2000, Sigma) in PBST for 1 hr at RT. Unbound secondary antibody was removed and positive bands were detected with a chemiluminescent reaction.

Sox9 shRNA knockdown

Cells were transduced with 20 μg of 10^6 cfu of lentiviral particles (Mission, Sigma) in 10% CCM for 48 hrs. The medium was then replaced with new medium without viral particles and puromycin (20 $\mu\text{g}/\text{ml}$) was added for genetic selection of successfully-transduced cells. The surviving cells were cloned and expanded for further experiments. For Sox9 knockdown, we used the following shRNA sequence: 5' CCG GAC CTT CGA TGT CAA CGA GTT TCT CGA GAA ACT CGT TGA CAT CGA AGG TTT TT 3'.

Genotyping

Genotyping was performed with the REDExtractN-Amp Tissue PCR Kit (Sigma). We used the following primers for genotyping:

Keratin18, Internal Control (wild type): Forward, 5' CACGTGGGCTCCAGCATT 3';
Reverse, 5' TCACCAGTCATTTCTGCCTTT G 3'
Transgenic band: Forward, 5' GCGGTCTGGCAGTAAAACTATC 3';

Reverse, 5' GTGAAACAGCATTGCTGTCACCTT 3'.

EDNRB: Forward, 5' AGGAGACTGAATGCAGACCAGC 3';

Reverse, 5' CATGTTACA GCTTGCTCCTGTG 3'.

Experiments with conditional knockout (cKO) mice

Animals were injected intraperitoneally for 3 days with either vehicle (90% v/v oil, 10% v/v 200 proof Ethanol) or Tamoxifen (20 mg/ml in vehicle). MI surgery was performed the day following completion of the last injection. Hearts were harvested 48 hours after MI and fixed and processed for serial cryosectioning. For each animal, 9 sections were analyzed, maintaining the same section position as accurately as possible across all animals. In each section, the epicardial region in 6 border zones adjacent to infarct (left ventricle), 3 infarct regions (left ventricle), and 3 distal regions (right ventricle) were captured as high magnification epifluorescent images. A blinded operator then quantified the different cell populations from randomized, coded images.

Antibody	Application	Primary Ab	Secondary Ab
Rabbit anti-CD104 (Bioss, bs10028-R)	FACS/MACS	1:500	1:1000 anti-Rabbit microbeads (Miltenyi Biotec)
Rabbit anti-WT1 (Santa Cruz)	ICC	1:100	1:800 Alexa 594 goat anti-Rabbit (Molecular Probes)
Rabbit anti-GATA4 (Santa Cruz)	ICC	1:250	1:800 Alexa 488 goat anti-Rabbit (Molecular Probes)
Rabbit anti- ETB _R (Sigma Aldrich)	IHC WB	1:200/ 1:500	1:500 Alexa 488 goat anti-Rabbit (Molecular Probes) 1:1000 Biotin conj, goat anti- Rabbit (Sigma Aldrich)
Rabbit anti-Sox9 (Santa Cruz)	ICC	1:250	1:500 Alexa 488 goat anti-Rabbit (Molecular Probes)
Rabbit anti-Keratin18 (Sigma Aldrich)	ICC/IHC	1:200	1:500 Alexa 488 goat anti-Rabbit (Molecular Probes)
Ms anti-pan-Keratin (Sigma Aldrich)	ICC	1:250	1:500 Alexa 488 goat anti-Rabbit (Molecular Probes)
Ms anti-Vimentin (Millipore)	ICC	1:250	1:500 Alexa 488 goat anti-Rabbit (Molecular Probes)
Rat anti-vWF (Dako)	WB	1:500	1:1000 Biotin conj, goat anti- Rabbit (Sigma Aldrich)
Ms anti-alpha SMA (Santa Cruz)	WB	1:1000	1:5000 Biotin conj, goat anti-Ms (Sigma Aldrich)
Ms anti-GAPDH (Abcam)	WB	1:1000	1:5000 Biotin conj, goat anti-Ms (Sigma Aldrich)
Rabbit anti-CD90 (BD Biosciences)	FACS	1: 500	1:1000 anti-Rabbit (Molecular Probes)

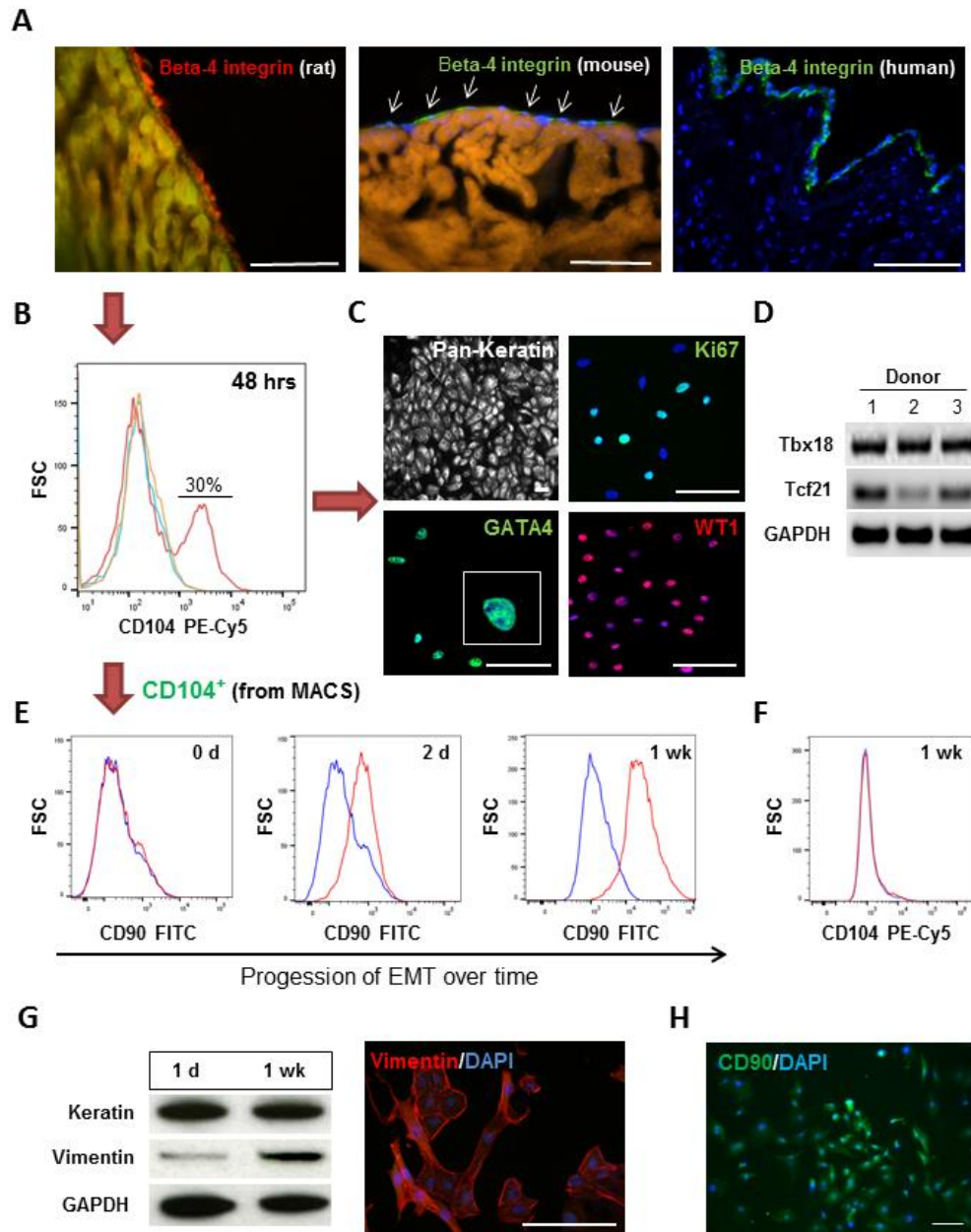


Figure 3- 1. CD104 identifies epicardial cells prior to EMT

(A) Immunohistochemistry for beta-4 integrin (CD104) indicating epicardial-restricted expression in the adult mammalian heart. Left to right: rat, mouse, human. Scale bars represent 100 μ m. (B) By cell surface phenotyping, 30% of the mixed cell population from atrial explants express CD104. (C) Immunocytochemical characterization of CD104⁺ cells. Isolated CD104⁺ cells are proliferative (Ki67) and express keratins (epithelial intermediate filament proteins) and transcription factors characteristic of

activated epicardial cells (GATA4, Wt-1). Scale bars represent 50 μ m. (D) RT-PCR assays for transcription factors (Tbx18, Tcf21) and GAPDH. (E-F) Epicardial cell EMT during 1 week in culture is characterized by gain of CD90 and loss of CD104 (F) (n=3 donors). (G) EPDC express increased Vimentin levels after EMT (n=3 donors). (H) After EMT, all EPDC express CD90. Scale bar represents 100 μ m.

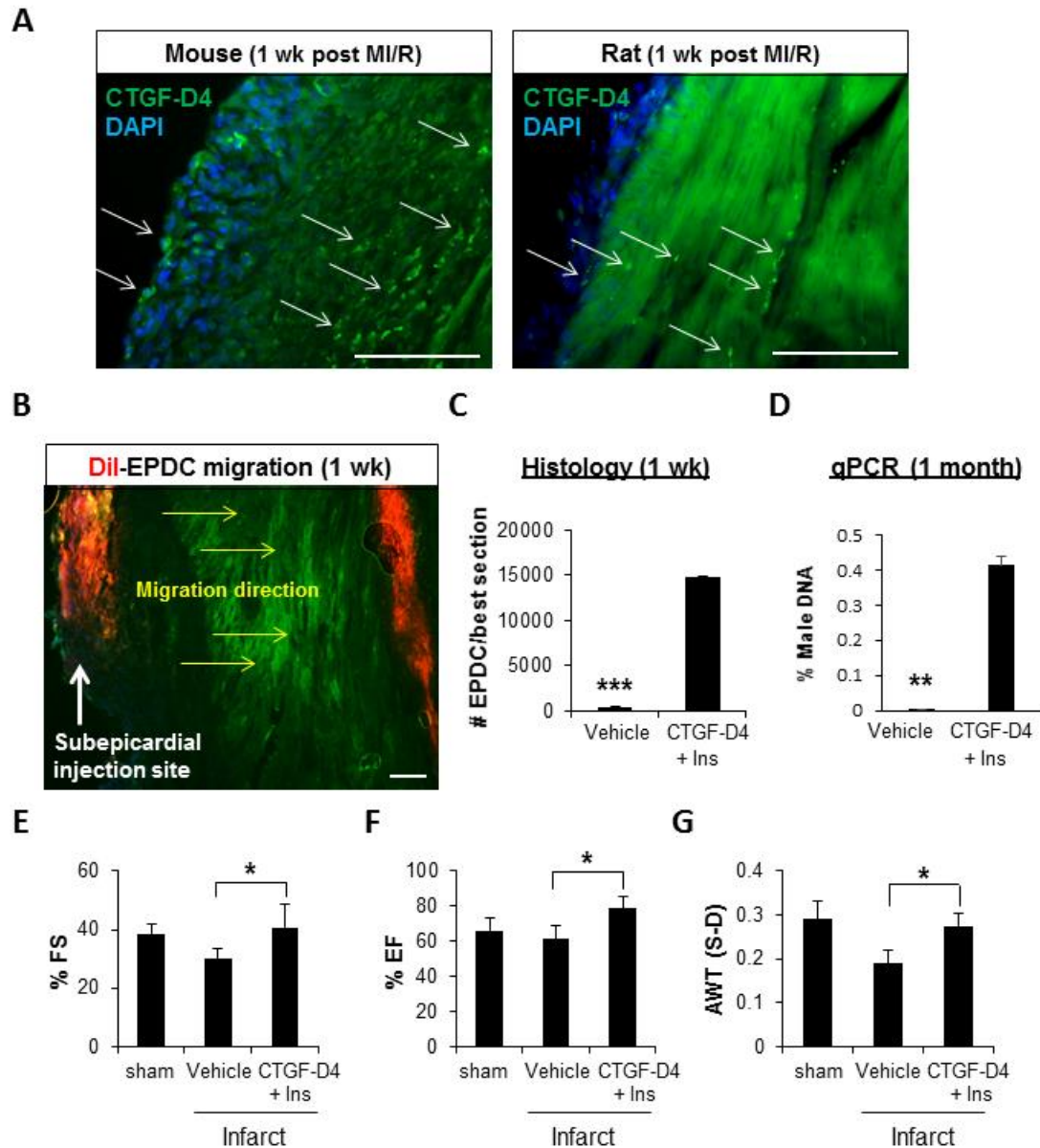


Figure 3- 2. CTGF-D4 promotes grafts of EPDC into the subepicardium after MI/R

(A) Expression of CTGF-D4 in the subepicardial cell layers and infarct border zone 1 week after MI with reperfusion (MI/R). (Left) adult mouse. (Right) adult rat. Scale bars represent 100 μ m. (B) Coronal cross section of adult rat heart after MI/R depicting engraftment at the subepicardial injection site (note residual DiI-labeled EPDC) and migration of DiI-labeled EPDC toward the ischemic myocardium. (C-D) Quantification of EPDC engraftment 1 week after transplant by counts of DiI-labeled cells (C) and 1 month after male EPDC transplant to female host by qPCR to detect the Y chromosome (D). (E-G)

Echocardiographic measures in animals 1 month after MI/R and EPDC transplant. (E) Fractional Shortening, FS. (F) Ejection Fraction, EF. (G) Anterior Wall Thickness, AWT (wall thickness in systole minus thickness in diastole). (C-G) Ins = Insulin. Error bars represent SD. Student's T-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bar represents 50 μm .

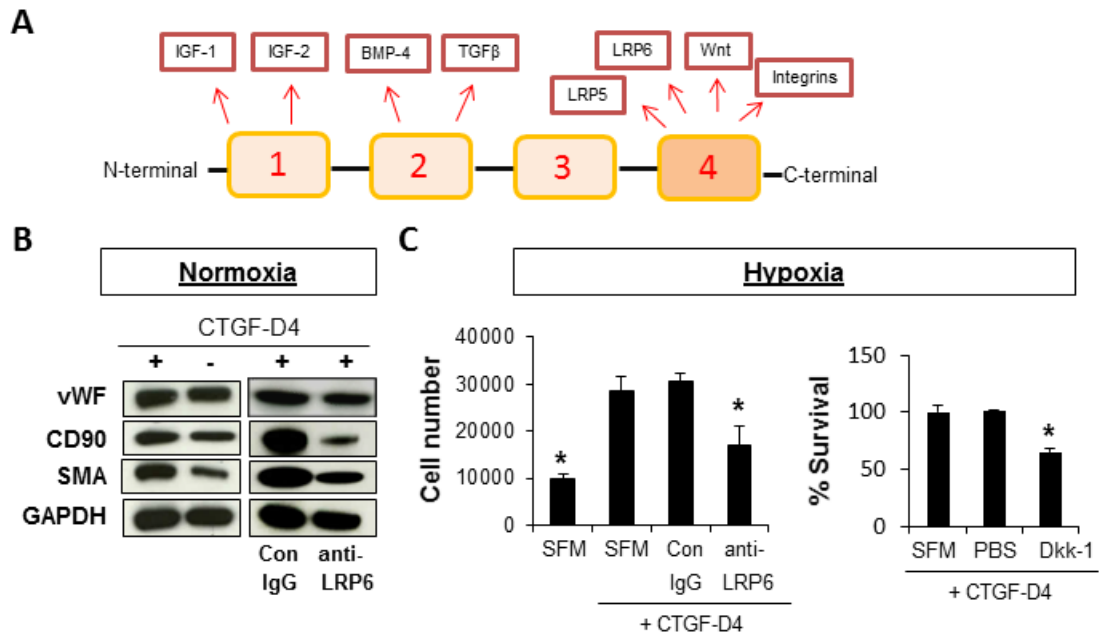


Figure 3- 3. CTGF-D4/LRP6 signaling promotes epicardial cell differentiation and survival

(A) CTGF has 4 distinct domains that interact with other cell ligands, cell surface receptors, and integrins. CTGF binds LRP6 via its N-terminal domain (CTGF-D4). (B) Treatment with CTGF-D4 promotes EPDC differentiation in an LRP-6-dependent manner. CD90 (cell adhesion, differentiation), vWF (endothelial cells), α -SMA (smooth muscle cells and myofibroblasts). (C) (Left) Exposure of primary EPDC to CTGF-D4 increased cell survival during hypoxia (48 hr), an effect blocked by neutralizing antisera to LRP6. (Right) Exposure of primary EPDC to Dkk-1 (20 ng/ml) prior to treatment with CTGF-D4 prevented CTGF-D4 mediated cell protection under hypoxia. (D) Design for neutralization experiment to assay requirement of LRP6 during EPDC engraftment after MI/R. Cultured EPDC from the same donor/dish were lifted, split, and separately dye-labeled prior to incubation with blocking antisera (anti-Lrp6) and CTGF-D4 or non-specific IgG (con IgG) and CTGF-D4. Note: Cells were incubated with antibodies prior to CTGF-D4. Equal proportions of EPDC were mixed and grafted together in a competitive engraftment assay. (E-E') Representative tissue sections 1 week after MI/R and cell transplantation. (E') Cell control with reciprocal dye labeling. (F) EPDC counts after 1 week of engraftment (n= 11). (G) qPCR data for Y chromosome at 1 month after engraftment (n= 3). Error bars represent SD. Student's T-test. * $p \leq 0.05$, ** $p \leq 0.01$. Scale bar represents 100 μ m.

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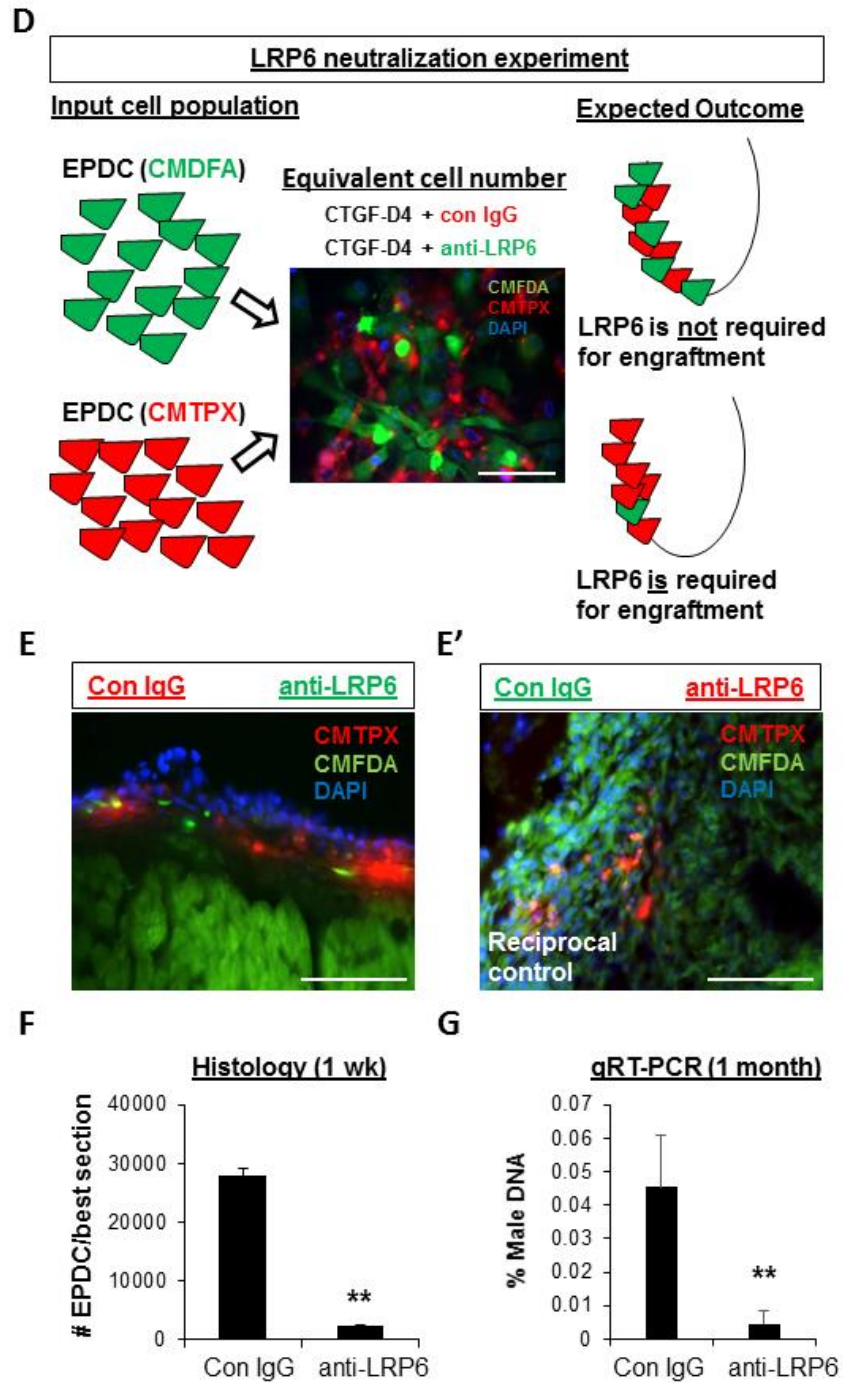


Figure 3-3. CTGF-D4/LRP6 signaling promotes epicardial cell differentiation and survival

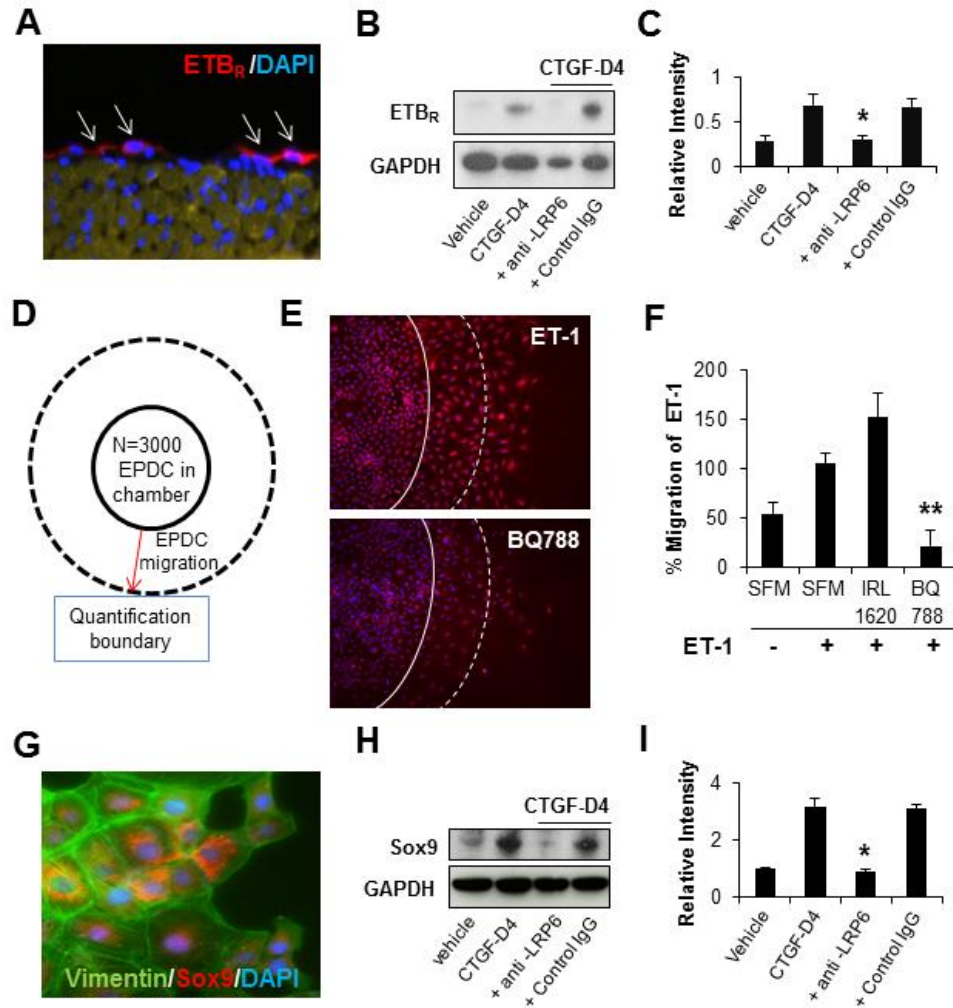


Figure 3- 4. CTGF-D4/LRP6/Sox9 signaling promotes ETBR and Sox9 expression in EPDC

(A) Epicardial cells on the heart surface express ETBR. Scale bar represents 100 μ m. (B-C) CTGF-D4 treatment increases ETBR levels in an LRP6-dependant manner. (C) Quantification of normalized band intensity for ETBR (n=6 donors per treatment group). (D) Design of “donut assay” to measure radial cell migration over time in “t” hours, from seeding location inside the donut (see solid line, center) to outside the donut (see dotted line, beyond outer edge). (E) Representative images of EPDC migration in donut assay (Red, DiI; Blue, DAPI). (F) Quantification of EPDC migration with addition of agonist or antagonist. Note: Data are normalized for total cell number. (G) After EMT, vimentin-positive EPDC express Sox9. Scale bar represents 50 μ m. (H) CTGF-D4 treatment increases Sox9 levels in an LRP6-dependant manner. (I) Quantification of normalized band intensity for Sox9 (n=5 donors per treatment group).

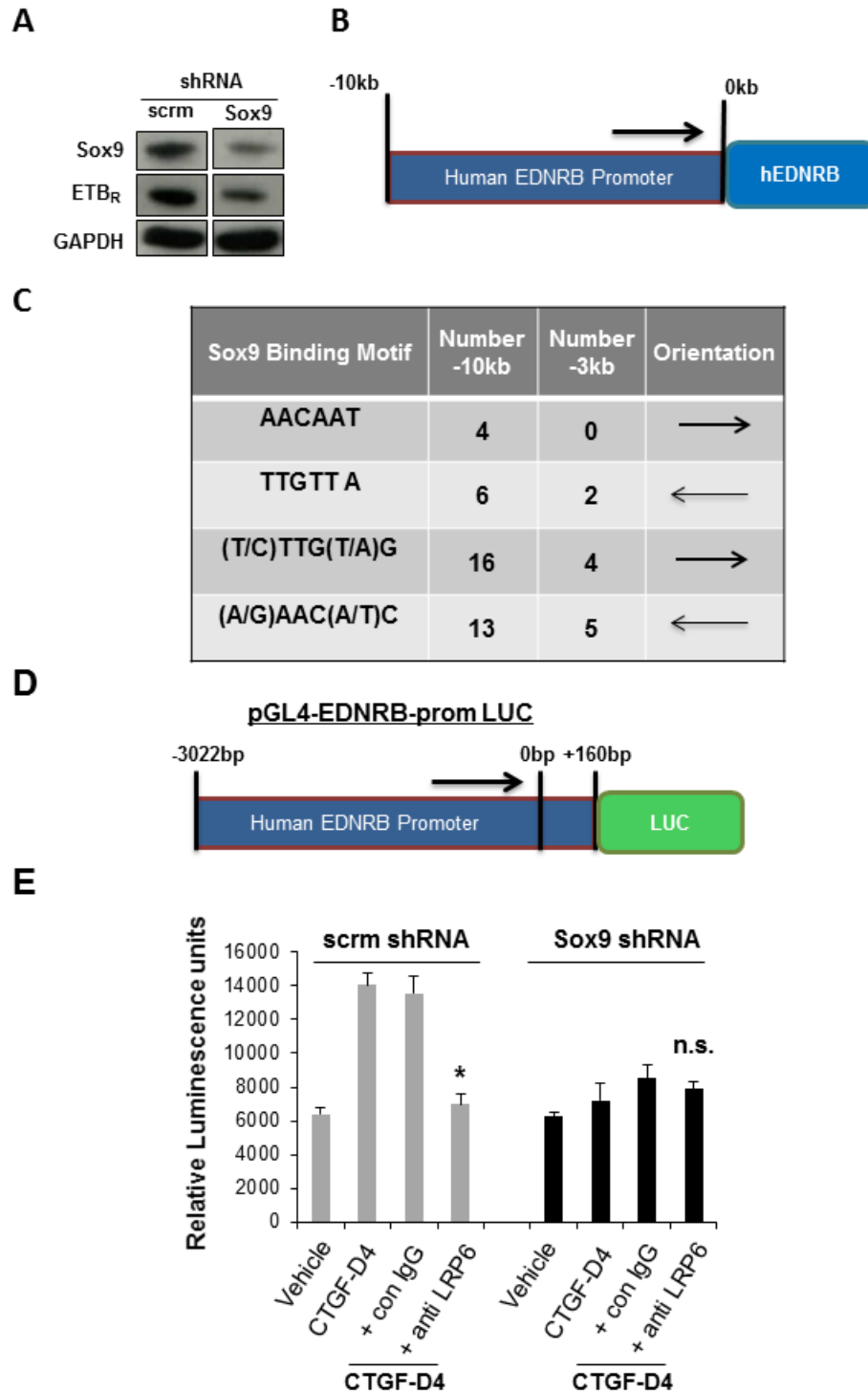


Figure 3- 5. CTGF-D4/LRP6/Sox9 signaling axis controls ETBR levels

(A) Sox9 knockdown in HEK293 cells. Sox9 and ETB_R levels are decreased in cells expressing Sox9 shRNA relative to those expressing scrambled shRNA (control). (B,C) Human promoter sequences for the *EDNRB* gene contain multiple putative Sox9 binding sites. Table summarizes the number of sites upstream of the transcriptional start site (0 kb) that match Sox9 binding motif AACAAAT or its reverse complement TTGTTA and motif (T/C)TTG(T/A)G or its reverse complement (A/G)AAC(A/T)C. Arrows in the last column of the table in (C) represent direction of promoter sequence in the genome. (D) Schematic of reporter construct for the human *EDNRB* promoter (pGL4-h*EDNRB*-prom) used for transfection experiments and luciferase activity assays. A 3.5 kb span of the human *EDNRB* promoter was cloned upstream of the firefly luciferase gene (LUC). +160bp indicates the position of the start codon. (E) Relative luciferase activity (luminescence units) of HEK 293 cells transiently-transfected with pGL4-human *EDNRB* promoter reporter construct. Error bars represent SD. Student's T-test. *p ≤ 0.05, **p ≤ 0.01. Scale bar represents 100 μm.

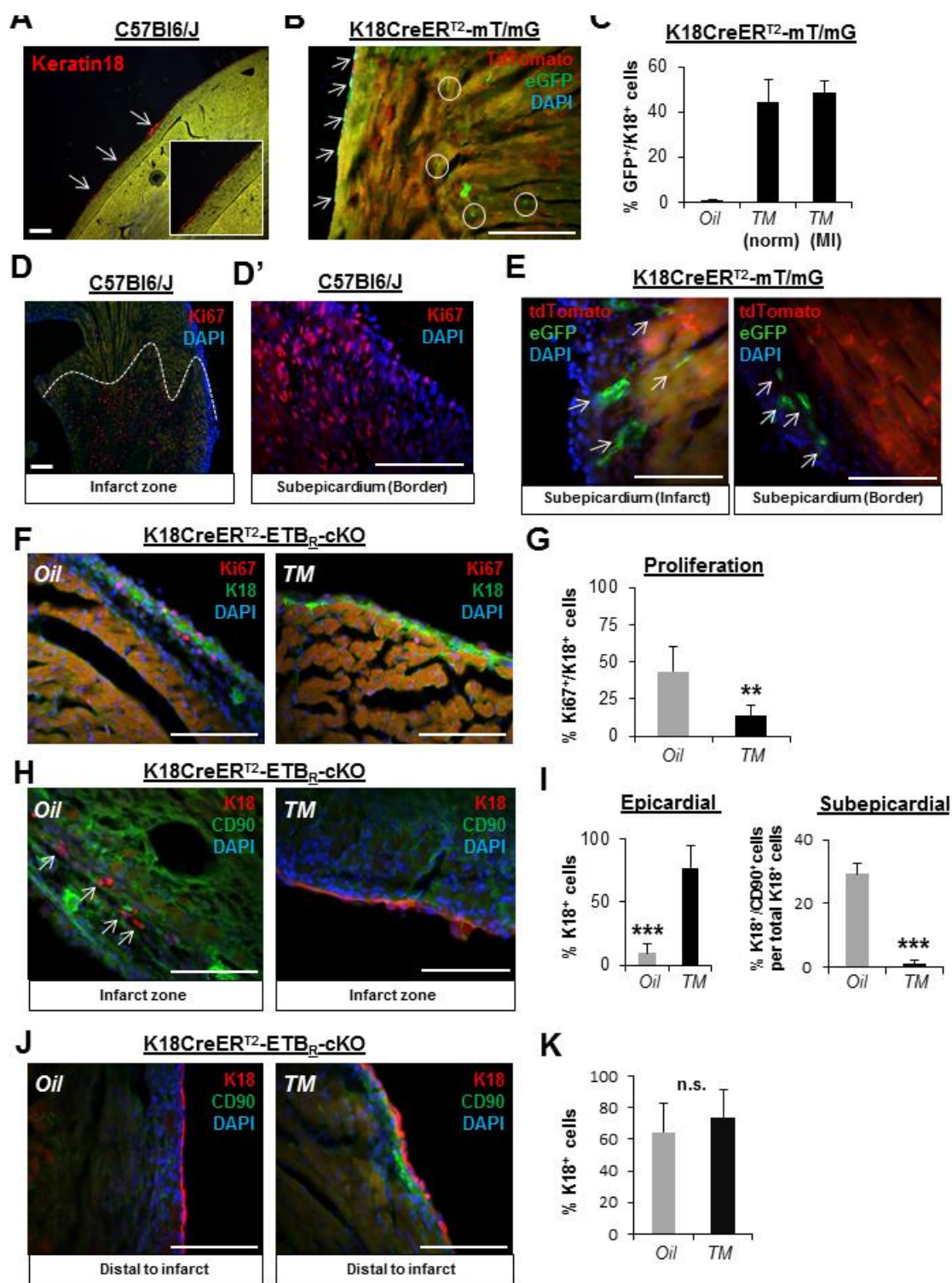


Figure 3- 6. ETB_R controls epicardial proliferation and migration

(A) In wildtype mouse heart, Keratin18 (K18) is expressed specifically by epicardial cells. Inset: Magnified view of epicardial surface. Scale bar represents 100 μm . (B) TM-inducible K18CreER^{T2}-mT/mG mice lineage-tag epicardial cells on the surface of the heart (GFP, arrows) and a population of EPDC in the myocardium (GFP, circles). Scale bar represents 100 μm . (C) Lineage-tagging efficiency as determined by quantification of GFP⁺ cells (from lineage-tagging), K18⁺ cells (from immunostaining), and GFP⁺/K18⁺ cells under homeostasis and after MI (n=3 animals per group). Note: Negligible recombination from oil injection. (D,D') Proliferating epicardial cells (Ki67⁺) 3 d after MI in the infarct zone (D, dotted line) and in the subepicardial regions bordering the infarct (D'). Scale bars represent 100 μm . (E) Lineage-tagged, GFP⁺ EPDC migrate into the myocardial regions of infarct (left) and border zone subepicardium (right) 3 d after MI. (F) Conditional knockout of epicardial ETB_R significantly decreased the number of proliferating EPDC (K18⁺/Ki67⁺) in the subepicardium 3 d following MI (n=6 mice per group). (G) In contrast to oil-treated controls, with ETB_R knockout, many GFP⁺ cells remained epicardial (on the heart surface) after MI/R. Furthermore, with ETB_R knockout, the number of migrating EPDC (K18⁺/CD90⁺) was significantly decreased in the subepicardium bordering infarct (n=6 animals per group). (H) K18⁺/CD90⁺ EPDC were not affected in either the oil- or in the TM-treated groups in epicardial regions distal to the infarct zone. For F-H, scale bars represent 100 μm . TM = tamoxifen. Note: Bright yellow staining in myocardium is background autofluorescence. Error bars represent SD. *p \leq 0.05, **p \leq 0.01.

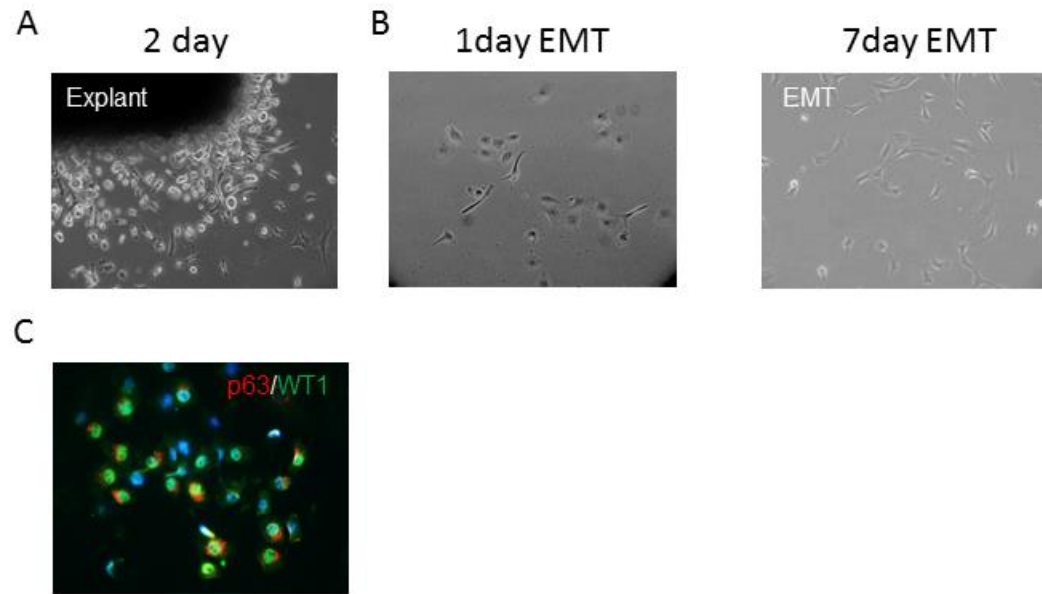


Figure 3- S 1. Epicardial EMT

(A) Primary epicardial explants in 10% FBS. (B) (Left) CD104 sorted primary EPDC after 1 day and 7 days of EMT. (C) A subset of CD104- positive cells stain for p63 and WT1 cells.

A

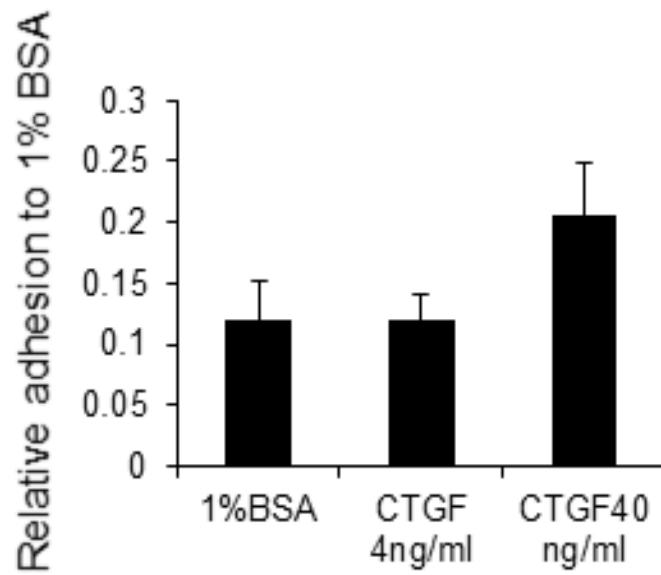


Figure 3- S 2. CTGF-D4 promotes cell adhesion

CTGF-D4 promotes the adhesion of primary EPDC on fibronectin coated cell culture surfaces.

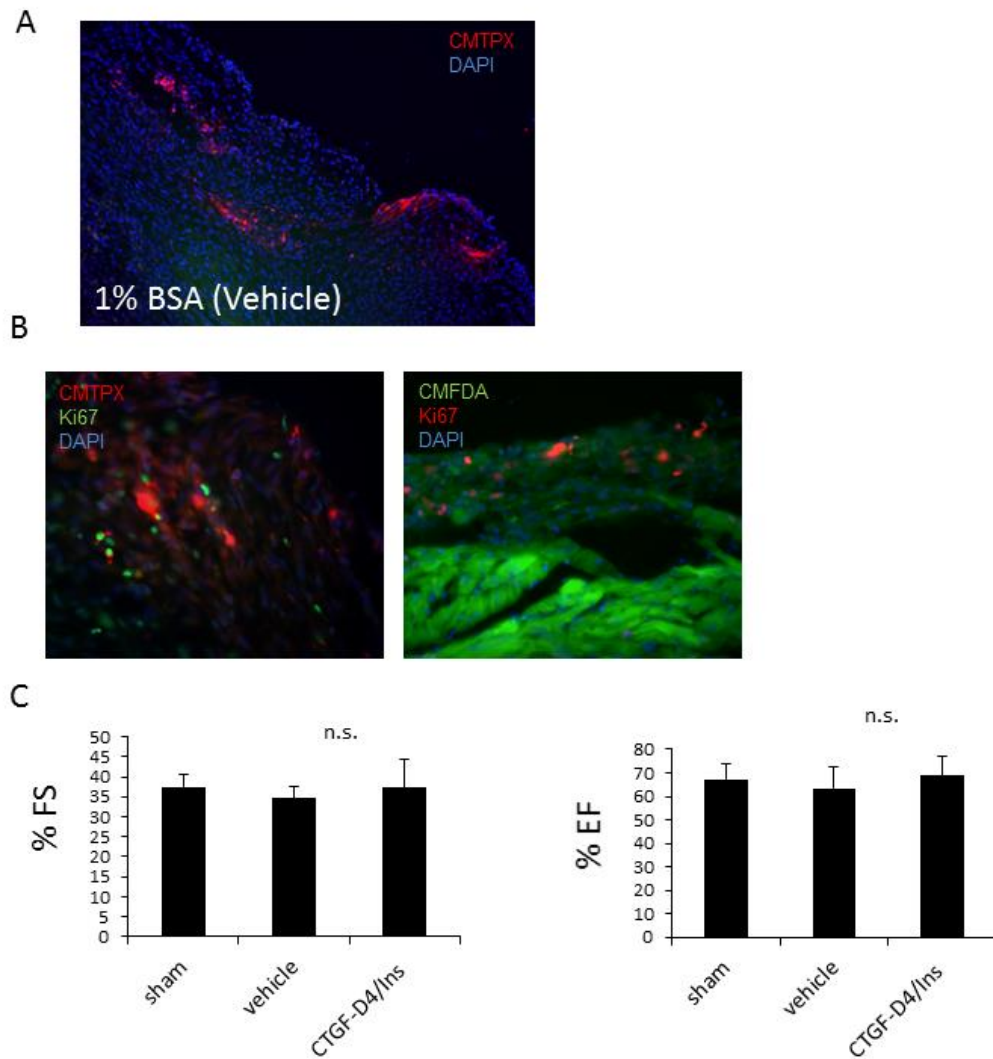


Figure 3- S 3. CTGF-D4 promotes Primary EPDC engraftment

(A) Primary EPDC engraftment with 1% BSA (vehicle control). (B) EPDC proliferation at 3 days following engraftment in CTGF-D4. Proliferating nuclei can be identified by Ki67 staining. (C) Fractional shortening and Ejection fraction measures of animals at 1 week after EPDC engraftment when compared to sham.

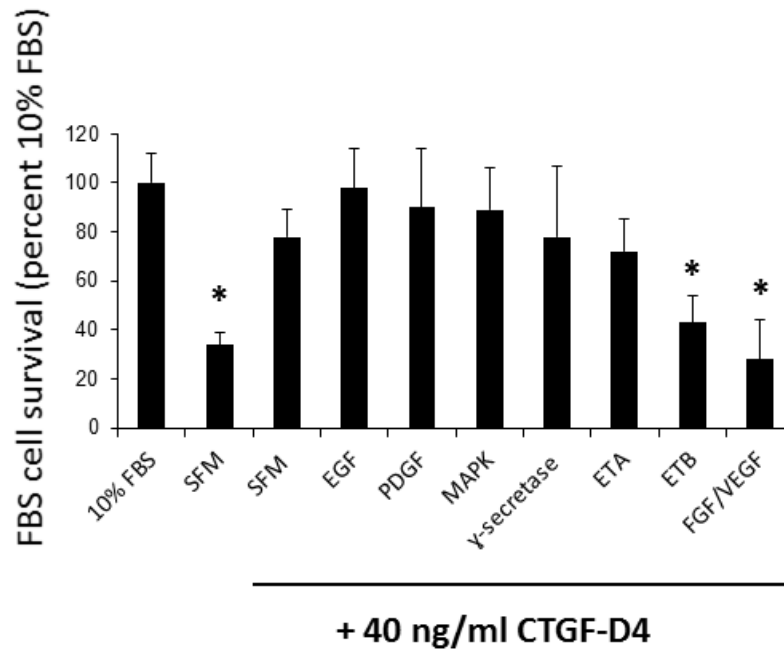


Figure 3- S 4. Pathway inhibitor screen

Cell protection assay in 1% hypoxia for 48 hours under the defined treatment conditions. Inhibitor screen treatments included complete culture medium (10% FBS), Serum free medium (SFM), EGF inhibitor (CAS 879127-07-8), PDGF inhibitor (CAS 347155-76-4), MAPK inhibitor (CAS 331662-51-2), Notch pathway inhibitor (gamma-secretase inhibitor: DAPT), Endothelin Receptor A inhibitor (BQ123) Endothelin Receptor B inhibitor (BQ788), FGF/VEGF inhibitor, (CAS 144335-37-5).

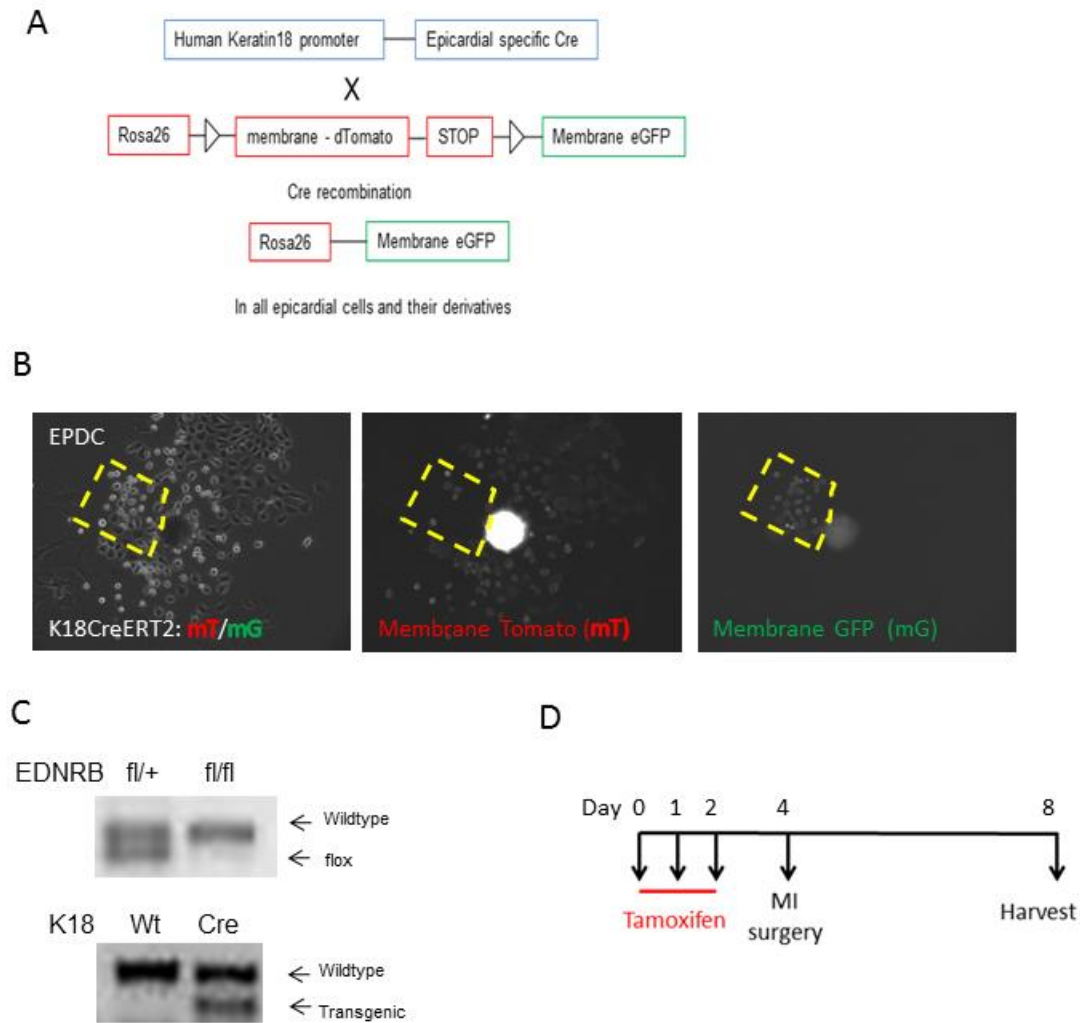


Figure 3- S 5. Transgenic K18CreER mice

(A) Transgenic K18CreER mouse breeding scheme with lineage reporter mT/mG mice. (B) 4-OH-TM induction of eGFP (mG) in cultured epicardial cells isolated from K18CreERT2:mTmG mice. Left, Phase contrast. Middle, TRITC channel (mT, red). Right, FITC channel (mG, green). Note: Yellow dotted line surrounds a group of K18+ mG cells. (C) Genotyping band patterns for K18CreERT² and Endothelin Receptor B flox/flox mice. (D) Timeline for Tamoxifen injections prior to MI injury in transgenic mice.

CHAPTER IV. ADULT EPICARDIAL CELLS CONTRIBUTE TO THE CAPPILARY NETWORK OF THE HEART DURING RUNNING EXERCISE

Introduction

During development, a subset of epicardial cells undergo epithelial to mesenchymal transformation to provide a population of migratory progenitors or epicardial- derived cells (EPDCs) (Lie-Venema et al., 2007; Munoz-Chapuli et al., 2002; Wessels and Perez-Pomares, 2004). The EPDCs migrate through the developing myocardium and secrete signaling factors that play a role in tissue morphogenesis and in ensuing the completion of cardiac development, which includes the proliferation and maturation of cardiomyocytes (Combs et al., 2011; Vega-Hernandez et al., 2011; Weeke-Klimp et al., 2010). The epicardial layer also contributes progenitor cells that terminally differentiate into a majority of the adventitial fibroblasts and vascular smooth muscle cells (Dettman et al., 1998; Gittenberger-de Groot et al., 1998; Perez-Pomares et al., 1998; Vrancken Peeters et al., 1999). Both of these cell types are critical to the structural and functional integrity of the developing blood vessels. In contrast, the potential of the epicardial precursor cells to differentiate directly into cardiac endothelial cells or myocytes (cardiomyocytes) is still under debate. Furthermore, contribution of the EPDCs towards any of the above differentiated cell lineages in adults needs to be established in a definitive manner.

To determine whether the epicardial layer functions as a source of progenitor cells in adults, we assessed the cellular contribution of EPDCs in response to a physiological demand for tissue growth or regeneration. To do so, we generated a novel

lineage-tracing mouse model to label and track epicardial cells on the cover of the heart and EPDCs that migrate throughout the myocardium. We performed lineage-tracing during chronic exercise, in which growth of the myocyte layer and capillary network was previously reported. Other groups demonstrated that exercise results in hypertrophic growth of the cardiomyocytes (by both enlargement and cell proliferation) (Abrahams, 1946; Towbin et al., 2015; Van Lier and Northup, 1957; Vanliere et al., 1965). Importantly, this form of cardiac hypertrophy is accompanied by an expansion of the capillary network due to increased demand for perfusion throughout the heart to meet the demand for cardiac output that results from exercise (Anversa et al., 1987; Cosmas et al., 1997; Laughlin et al., 2012; Mall et al., 1990; Saltin et al., 1986). For lineage-tracing epicardial dynamics and fate, we found that the exercise model provides several advantages over models of experimental myocardial infarction, where necrotic signals and extensive tissue death limit our ability to thoroughly evaluate the cellular contribution of the epicardium to regeneration.

Methods

The Cre-loxP technology was utilized to lineage-tracing adult epicardial cells from the cover of the heart. Keratin18 (epithelial intermediate filament protein) is expressed in the heart exclusively by epicardial cells and was used to drive the expression of “Cre” a bacterial recombinase enzyme. In the transgenic mouse model, Cre remains physically associated with estrogen receptor (ER) thereby limiting its expression to the cytoplasm throughout the lifetime of the animal (K18CreERT2). Translocation of Cre to the nucleus to carry out genetic recombination function was facilitated by administration

of Tamoxifen (TM), an estrogen agonist. When K18CreERT2 animals were crossed with reporter mice (mT/mG or YFP), in the presence of TM, Cre mediated the excision of a STOP codon flanked by loxP sites (its chemical substrate) and enabled the expression of a downstream fluorescent reporter gene (GFP or YFP respectively). Subsequently, the resulting experimental animals permanently expressed GFP or YFP fluorescent markers in epicardial cells and EPDCs throughout the heart.

Following three days of TM injection, male mice (6-8 weeks of age) were housed in shoe-box sized cages and either provided with a running wheel (exercise) or not (non-exercise) for the entire duration of the experiment (8 weeks) (n= 20 per group). Animals were fed a normal diet and exercised mostly during their wake hours at night. After 8 weeks, echocardiography was performed, followed by tissue harvest for histology as previously described.

Results

To initially determine that the lineage-tagging system was operational, we examined mice early after running, and observed that epicardial cells on the heart covers of K18CreERT2:mTmG mice were GFP⁺. At 8 weeks after exercise, a number of these cells migrated through the myocardium were detected in a pattern similar to the capillary network of the heart (Figure 1). The vascular-like patterning of the lineage-tagged epicardial derivatives was similar through both the ventricular and atrial chambers.

Closer examination of tissue from K18CreERT2:YFP mice and double staining CD31, a marker for differentiated endothelial cells, indicated a widespread distribution of YFP⁺/CD31⁺ cells throughout the heart (Note: Due to constitutive expression of

membrane Tomato in the mT/mG reporter mice, we utilized YFP lineage trace in order to be able to use the red fluorescent channel for staining lineage markers). Intriguingly, the YFP⁺/CD31⁺ cells also appeared as mostly individual capillaries or smaller networks of capillaries with short branches with few or no YFP⁺/CD31⁺ in the larger arterioles or coronary arteries (Figure 2). Quantification of the lineage-tagged endothelial cells revealed a significantly greater number of epicardial-derived YFP⁺/CD31⁺ capillaries in the exercised group of animals compared with the group that was not exercised (Figure 3). Of interest, we observed only rare epicardial- derived YFP⁺ cells that differentiated into smooth muscle cells, fibroblasts or cardiomyocytes both in the exercised and non-exercised group of animals.

To determine whether there were differences in the proliferation and/or activation of the epicardial layer on the surface of the heart in response to running exercise, we analyzed the lineage-tagged cells YFP⁺ cells for expression of Ki67 (proliferation marker) (Figure 5; $p = 0.71$) and WT-1 (activation marker) (Figure 6; $p = 0.58$). Interestingly, we did not observe significant increase in expression of either of markers as a result of prolonged exercise over 8 weeks (Figure 4). However, in agreement with previous reports (Borer et al., 1983; Nishimura et al., 1980; Perez-Gonzales et al., 1981; Perrault et al., 1986), we did observe a significant improvement in cardiac function, including parameters of ejection fraction, fractional shortening in the animals that were exercised, compared to the non-exercised group (Figure 4; $n = 10$ per group, $p < 0.05$).

Summary

These results demonstrate that the epicardial layer contributes to the capillary network after running exercise. In contrast to injury models such as myocardial infarction, where fibroblast differentiation predominates, after exercise other differentiated lineage-traced cells were rare. Further understanding of the molecular mechanisms that control endothelial differentiation of epicardial cells could have profound impact on the application of epicardial cells in cellular therapies for cardiac regeneration.

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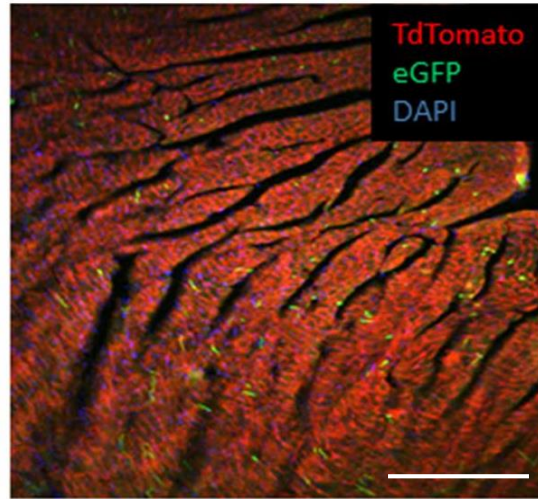


Figure 4- 1. Keratin 18 lineage-labels epicardial cells and its derivatives

K18CreER^{T2}:mT/mG labels epicardial cells on the surface of the heart as GFP⁺ at 1 week after TM injection. GFP⁺ cells migrate into the myocardium and demonstrate a capillary network like distribution throughout the heart. Scale bar=50 μ m.

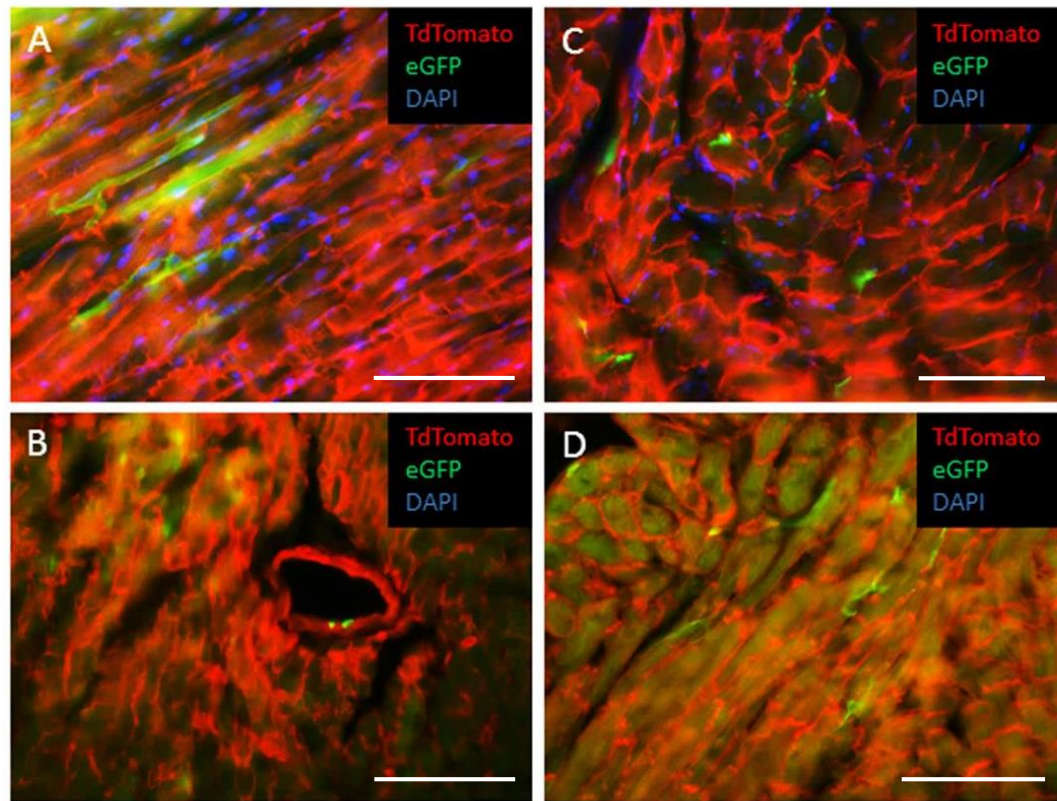


Figure 4- 2. Epicardial-derivatives differentiate into endothelial cells after running exercise

(A,C,D) K18CreER^{T2};mT/mG labels epicardial cells on the GFP⁺ through the capillary network in the heart that are closely associated with the cardiomyocytes, 8 weeks after running exercise. (B) Few GFP⁺ cells migrate into the myocardium in a representative non-exercised animal. Scale bar = 100 μm.

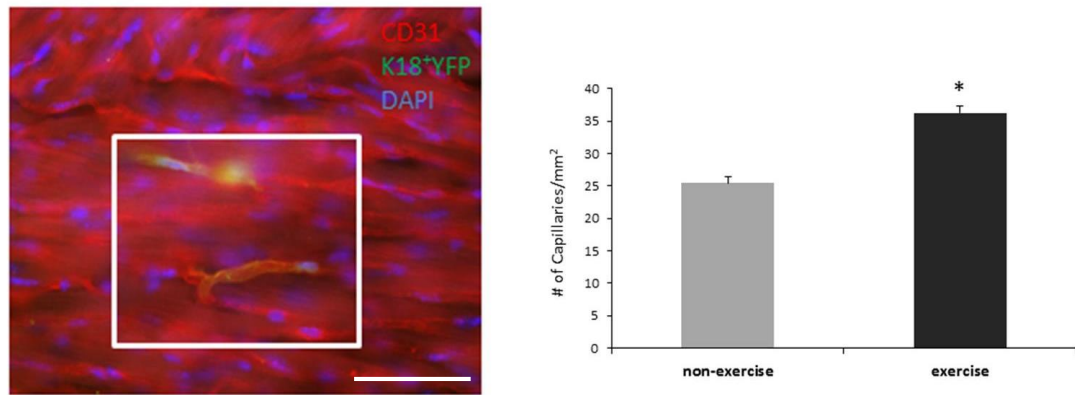


Figure 4- 3. Increase in lineage-labeled capillary density after running exercise

(Left) High magnification image of K18CreER^{T2}:YFP animals label double positive with endothelial marker CD31. (Right) Quantification of the number of lineage-traced capillaries in heart sections in exercised and non-exercised animals. Scale bar = 100 μ m.

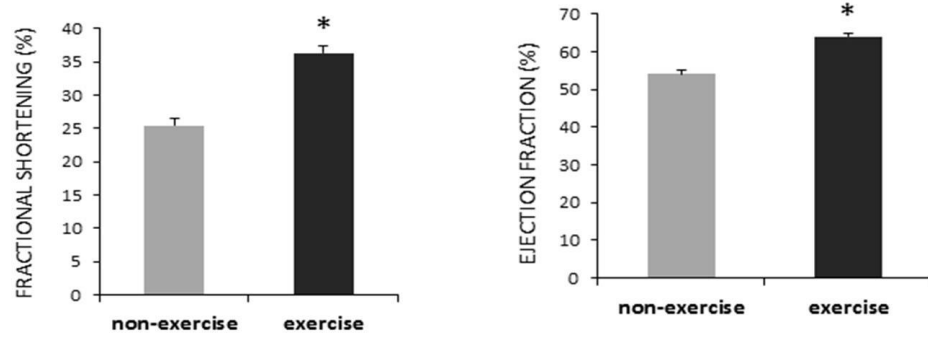


Figure 4- 4. Improvement in cardiac function after 2 months of running exercise

Improvement in cardiac function after 2 months of running exercise. (Left) Fractional shortening. (Right) Ejection fraction. (n = 10 per group). * p < 0.05.

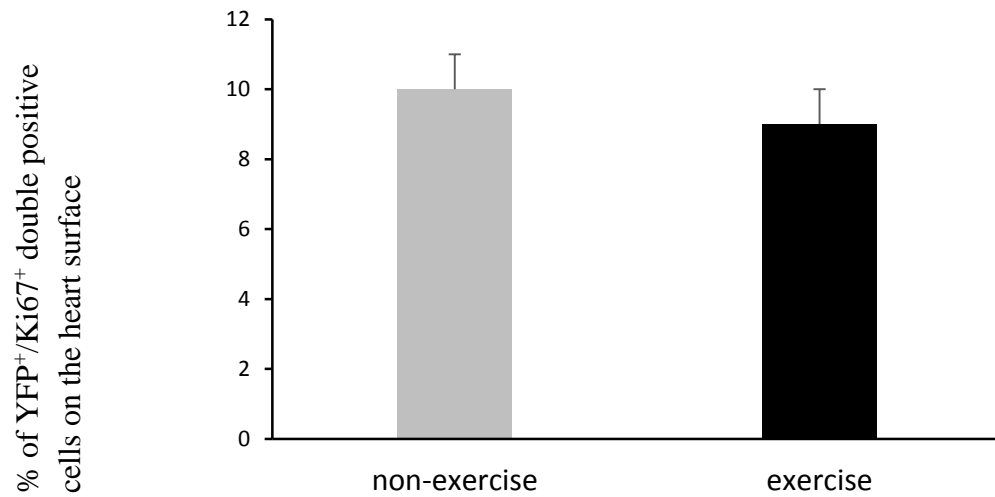


Figure 4- 5. Epicardial proliferation on the cover of the heart does not increase during running exercise

Number of lineage-labeled epicardial cells that are Ki67-positive on the heart cover of exercised animals vs non-exercised animals. Note, cells were counted from (n= 10) animals per group, across multiple cross sections of the heart of each animal, from apex to base.

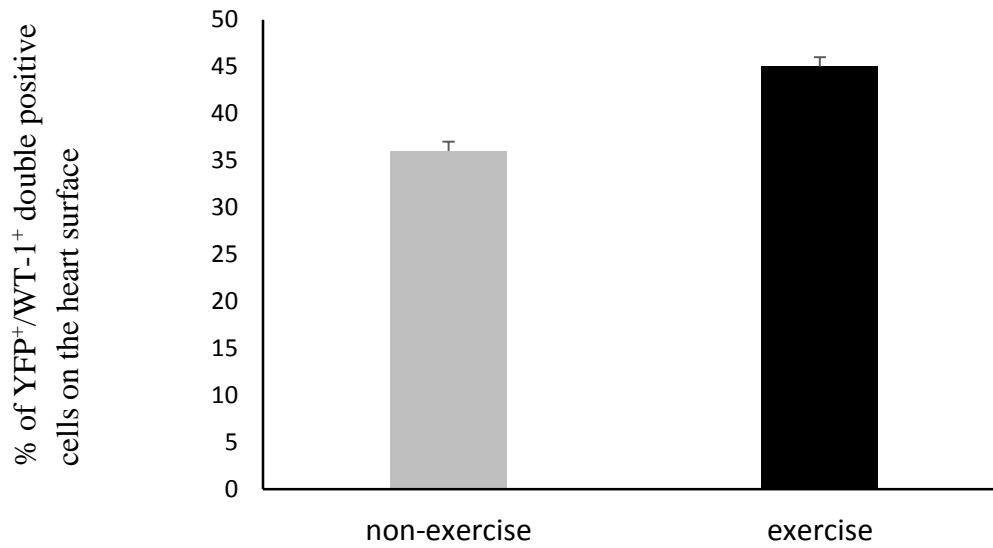


Figure 4- 6. Epicardial cells are not activated on the cover of the heart in response to running exercise

Number of lineage-labeled epicardial cells that are WT-1- positive on the heart cover of exercised animals vs non-exercised animals. Note, cells were counted from (n= 10) animals per group, across multiple cross sections of the heart of each animal, from apex to base.

**CHAPTER V. HUMAN EPICARDIAL CELL-CONDITIONED
MEDIUM CONTAINS HGF/IgG COMPLEXES THAT
PHOSPHORYLATE RYK AND PROTECT AGAINST VASCULAR
INJURY**

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Abstract

Aim: Profile paracrine activity of human Epicardial-Derived Cells (hEPDC) to screen for secreted vaso-protective factors and develop therapeutics to treat vascular reperfusion injury.

Methods and Results: Epicardial cells support cardiac development, repair and remodeling after injury in part, through paracrine activity. We hypothesized secreted ligands from hEPDC would protect vascular integrity after MI with reperfusion. During simulated ischemia in culture (24-48 hr), concentrated hEPDC-conditioned medium (EPI CdM) increased survival of primary cardiac endothelial cells. In a rat MI model, EPI CdM treatment reduced vascular injury *in vivo* after reperfusion. By phospho-Receptor Tyrosine Kinase (RTK) arrays, ELISA, and neutralizing antibody screens we identified Hepatocyte Growth Factor (HGF) as a key vaso-protective factor in EPI CdM. Unexpectedly, we observed that ~30% of HGF in EPI CdM formed complexes with polyclonal IgG. Following reperfusion, preparations of HGF/IgG complexes provided greater vascular protection than free HGF with IgG. HGF/IgG complexes localized to blood vessels *in vivo* and increased HGF retention time after administration. In subsequent screens, we found that “related to tyrosine kinase” receptor (RYK) was phosphorylated after exposure of cardiac endothelial cells to HGF/IgG complexes, but not to free HGF with IgG. The enhanced protection conferred by HGF/IgG complexes was lost after antibody blockade of RYK. Notably, the HGF/IgG complex is the first “ligand” shown to promote phosphorylation of RYK.

Conclusions: Early treatment with HGF/IgG complexes after myocardial ischemia with reperfusion may rescue tissue through vasoprotection conferred by c-Met and RYK signaling.

Introduction

The epicardium, a specialized epithelial cell layer that covers the heart, is essential to cardiac development, structure and function.^{1,2} During development, a subset of epicardial cells undergo epithelial to mesenchymal transformation (EMT) into EPicardial-Derived Cells (EPDC), precursor cells that invade the subepicardium and myocardium and contribute to the subepicardial and coronary vasculature and the pool of interstitial fibroblasts.³⁻⁵ EPDC interact with neighboring cells such as cardiomyocytes and Purkinje fibers and affect their proliferation and function,^{3,6,7} in part through paracrine activity.⁸ In adults, epicardial cells play important roles in repair and remodeling after cardiac injury. Ventricular regeneration in zebrafish requires epicardial cell proliferation, EMT, invasion and subsequent neovascularization of myocardium.⁹ Factors secreted by EPDC of adult mice were reported to increase angiogenesis after myocardial infarction (MI); these paracrine effects correlated with decreased infarct size and improved cardiac function.¹⁰

Historically, the field of cardiac regenerative medicine has focused on transplantation of stem/progenitor cells to reduce infarct size and improve cardiac function through direct replacement of damaged myocytes and vascular cells.^{11,12} However, numerous reports now indicate paracrine effects were responsible for much of the observed benefits of cell therapy. Stimulation of angiogenesis is frequently reported due to higher numbers of blood vessels observed in tissues/organs treated with stem/progenitor cells^{13,14}, but few studies have considered rescue and/or repair of pre-existing blood vessels as opposed to angiogenesis. Importantly, secreted factors that

protect microvascular endothelial cells early after reperfusion may reduce vascular leak and infarct expansion after MI. To better understand the role of vaso-protection (rescue) in the paracrine benefits of cell transplantation, we isolated human EPDCs (hEPDC) and investigated the ability of hEPDC-conditioned medium (EPI CdM) to protect the vasculature early after cardiac ischemia with reperfusion. Through screens of EPI CdM, we identified novel HGF/IgG protein complexes that enhance vaso-protection, in part, by activating a Wnt co-receptor called “related to tyrosine kinase” (RYK). Our results suggest treatment with EPI CdM or HGF/IgG complexes may reduce reperfusion injury and infarct expansion in patients with MI.

Methods

A detailed description of methods is available in the online supplementary materials.

Human epicardial derived cell-conditioned medium (EPI CdM)

Conforming with the principles outlined in the Declaration of Helsinki, and with patient’s informed consent, right atrial appendages were obtained during cardiac bypass surgery. The right atrial biopsies were used to derive primary cultures of hEPDCs. After EMT, passage 2 hEPDCs were seeded and grown in 150 cm² dishes (Nunc) in Claycomb base medium supplemented with 10% FCS (lot selected for rapid growth of hMSCs, Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Mediatech Inc., Hendron, VA). When the cells reached 80-90% confluence, the plates were washed twice with PBS and serum-free MEM (MEM) was placed on the cells (20 mls per plate). After 48 hours of incubation, the EPI CdM was

collected, filtered (0.2 μ m PES membrane, Nalgene MF75, Rochester, NY), and concentrated to 10-fold or 30-fold with a Labscale™ TFF diafiltration system with a 5 kD cut-off filters (Millipore, Bedford, MA) or with Amicon Centricon filters (Millipore) with a 5 kD cut-off. One ml vials of EPI CdM were frozen and stored at -80°C. Some 1x unconcentrated EPI CdM was aliquoted and reserved for ELISAs and cell protection assays with primary human cardiac endothelial cells. ELISAs for human growth factors were performed with commercially available kits (R and D systems).

Myocardial ischemia-reperfusion surgery and treatment with EPI CdM

All animal procedures conformed to the Guide of the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 2011) and to the IACUC guidelines for animal care approved by the University of Vermont. Fischer 488 rats (males, 7 weeks of age) were weighed, shaved, anesthetized under 4% isoflurane, and endotracheally-intubated. Rats were ventilated at a respiration rate of 65 beats per min under a peak inspiration pressure of 15 cm H₂O (Kent Scientific). Body temperature was maintained at 37°C with a heated pad (Gaymar). Through a dermal incision, a blunt dissection of the fascia was performed and the intercostal muscles were separated. The heart was exposed by retraction of the pericardium to expose the left anterior descending coronary artery (LAD). The LAD was then encircled with 6-0 nylon suture. To facilitate release of suture at the time of reperfusion, a 0.4 cm length of PE10 tubing was placed over the LAD. The LAD was then occluded; this was confirmed by blanching of the anterior free wall of the LV. Animals were allowed to recover off the ventilator. After 2 hours, the rats were re-

intubated under anesthesia, ventilated, and the chest wall was re-opened. Hearts were exposed to reveal the suture, which was released, and reperfusion was visually confirmed by blood flow through the LAD and “re-pinking” of the blanched area. For each rat, we injected 1 ml of MEM, EPI CdM, or other treatments steadily over a period of 1.5 min through LV wall into the ventricle lumen (intra-arterial, 30.5 gauge needle). After the injection, the chest wall was closed and the rats recovered in their cages for 24 hrs prior to euthanization and tissue harvest. Under anesthesia, the chest of sham-operated animals was opened (twice) to visualize the intact pericardium, corresponding to the times of ischemia and reperfusion surgeries. No further manipulations or treatments were performed on the sham animals.

Measurement of vascular permeability *in vivo*.

At 22 hours post-ischemia, animals were injected with 0.5 ml FITC-albumin (tail vein, 5 mg/ml). After 2 hours, rats were anesthetized, perfused with 50 ml sterile PBS to wash out circulating FITC-albumin from blood vessels, and whole hearts were excised into sterile PBS. To isolate the left ventricles with septum, the adventitial tissue, major vessels and left and right atria were separated. Hearts were then homogenized in PBS (2 ml/gm wet weight of tissue) using a Polytron dispersing tool (Euro Turrax T206 IKA Labortechnik; Dispersing Tool S25N-10G, outer diameter 10 mm; IKA Works Inc., Wilmington, North Carolina, USA) and centrifuged at 16,000 x g for 20 min. The soluble fraction was separated and 100 µl volumes were aliquoted to determine the amount of FITC extravasated into myocardial tissue. Fluorescence readings were measured in

duplicate at 480 nm excitation and 520 nm emission wavelengths on an HT Synergy plate reader (BioTek Instruments, Winooski, VT).

Preparation of HGF/IgG complexes

Recombinant human HGF was diluted to a working concentration of 10 µg/ml in sterile PBS. Mixed polyclonal IgG (non-specific) from human serum was diluted to 14 µg/ml. The IgG was mixed with diluted HGF in a total volume of 10 ml (1:1 molar ratio; HGF:IgG). The mixture was then concentrated 40-fold (from 10 ml to 250 µl) using a centricon device (Centricon Plus-70 Centrifugal Filter, Ultracel-PL Membrane, 30kDa, Millipore). This concentrated mixture was diluted in PBS to give final HGF:IgG doses of 1x or 10x. Different concentrations of HGF were then used for treatment studies either in the free “uncomplexed” form or as HGF/IgG complexes.

Statistical Analysis

Comparisons of data from individual control and treatment groups were made by 2-tailed Student's t test. For studies with comparison of multiple groups, we performed one way ANOVA with Bonferroni post-hoc testing. Values of $p \leq 0.05$ were considered statistically significant.

Results

Isolation of adult human epicardial progenitor cells and EMT into precursor cells

The epicardial layer from adult human right atrial heart tissue consisted of a single layer of epithelial cells that stained positive for keratin, epithelial “intermediate filament protein” (Figure 1A) and negative for vimentin (mesenchymal, Figure 1A). Human

epicardial cells were isolated from right atrial appendages commonly removed to place a cardiopulmonary bypass cannula as part of cardiac surgery. The cells were successfully isolated from 12 out of 13 donors (Supplemental Table 1). We developed methods to isolate proliferating epicardial progenitor cells from right atrial appendages (for details see Supplemental Materials and Methods). Explant cultures were generated from minced right atrial appendage (Figure 1B). Once fibroblast outgrowth reached about 70% confluence, the medium was switched to a low serum stem/progenitor cell growth medium containing LIF, EGF, and bFGF. Under these conditions, floating spheroids and bunches of cells formed and were collected, placed into new culture dishes and expanded (Figure 1C). The epicardial progenitor cells had an epithelial morphology and expressed keratins (Figure 1D). Differentiation (EMT) of the epicardial progenitor cells into a mixture of transitory-amplifying precursor cells (hEPDC) was induced by culture in medium containing 10% fetal calf serum (Figure 1D). During EMT into precursor cells, the progenitor cells downregulated their expression of keratins and expressed vimentin (Figure 1D). By RT-PCR, we detected several mRNAs for markers of EMT: Snail, Slug, Twist and Smad1 (Supplemental Figure 2A). In addition, the human precursor cells expressed transcription factors reported to be expressed by murine epicardial cells: WT1 (cardiac development)^{15,16}, GATA4 (myocardial differentiation and function)¹⁷, TCF21 (epicardial EMT, cardiac fibroblast specification)¹⁸, and RALDH2 (enzyme for retinoic acid synthesis)¹⁹ (Figure 2A). Expression of mRNA for several epicardial-related transcription factors decreased as the cells differentiated (see GATA5, WT1, Isl-1, and Tbx18; Figure 2B)²⁰. In contrast, expression of mRNA for smooth muscle actin and von Willebrand Factor (vWF) increased (Figure 2B), indicating that some hEPDC might be

differentiating into vascular smooth muscle cells or myofibroblasts and others into endothelial cells, respectively. At 2 weeks after EMT, by immunocytochemistry, we detected a few cells that were positive for smooth muscle myosin or vWF, confirming that some of the cells were precursors of smooth muscle cells and endothelial cells, respectively. The majority of epicardial-derived cells were positive for prolyl-4-hydroxylase, an enzyme involved in collagen biosynthesis and a marker of fibroblasts (Supplemental Figure 2B). Cell surface phenotyping demonstrated that the hEPDC were negative for hematopoietic and endothelial cell surface markers such as CD31, CD34, CD45, and c-kit, and also the vascular pericyte marker NG2. Multiple cell surface antigens typical of bone marrow MSCs such as CD105, CD90, CD73, CD54, CD49d, CD44, and CD29 were expressed by hEPDC after EMT (Supplemental Figure 3 and data not shown).

For the second isolation method, epicardial tissue was carefully dissected from the surfaces of human right atrial appendages and cultured directly (Supplemental Figure 1 and Supplemental Materials and Methods). Comparison by immunocytochemistry, cell surface phenotyping, and ELISA data indicated that the precursor cells derived from EMT did not differ whether the cells were rapidly induced to undergo EMT or were maintained for several weeks as epithelial progenitor cells and then induced to undergo EMT.

EPI CdM treatment promotes vascular integrity *in vivo*

To determine whether human EPI CdM could provide vascular protection *in vivo*, adult male Fischer rats underwent 2 hr of transient ischemia followed by 24 hr of reperfusion.

They were then treated with either MEM (vehicle control) or EPI CdM at the time of reperfusion. Twenty four hours after ischemia/reperfusion we quantified the amount of FITC-albumin extravasation in each treated animal by normalizing it to the level of extravasation in sham-operated animals. On tissue sections, we observed a greater level of FITC-albumin extravasation from vessels in the infarcted region from the left ventricle (LV) compared with no intact vessels outside the region of infarction, including the right ventricle (RV) (Figure 3A). Furthermore, heart homogenates from MEM-treated rats had 37.8% greater FITC fluorescence than homogenates from rats treated with EPI CdM. The amount of extravasated FITC-albumin in the MEM-treated group was $527.4\% \pm 109.33$ of sham, whereas in the EPI CdM-treated group it was $359.7\% \pm 78.82$ of sham ($p \leq 0.05$, $n=5$ per group; Figure 3B).

To investigate vascular effects of EPI CdM treatment, we performed immunoblotting on left ventricular homogenates. VE-Cadherin is a key junctional protein involved in maintenance of endothelial barrier integrity and the level of phosphorylated VE-Cadherin (pVE-Cadherin) is a useful indicator of increased vascular permeability.²¹ At 24 hr after MI, reperfusion, and treatment, the level of pVE-Cadherin (pY⁶⁵⁸) was significantly higher in the MEM-treated group of animals than in the EPI CdM-treated group ($p \leq 0.01$, $n=3$; Figure 3C). To examine the relative effects of EPI CdM treatment on vascular endothelial cells and smooth muscle cells, we next compared the levels of CD31 (PECAM1, endothelial marker) and smooth muscle alpha actin (α -SMA) in LV homogenates from the MEM and EPI CdM treatment groups. Consistent with enhanced endothelial cell survival, we observed significantly higher levels of CD31 in animals treated with EPI CdM compared with MEM-treated controls ($p \leq 0.05$, $n=3$; Figure 3C).

In contrast to the CD31 results, we observed equal amounts of α -SMA for the MEM and EPI CdM treatment groups (Figure 3C), indicating that EPI CdM protected endothelial cells.

EPI CdM protects human cardiac endothelial cells during simulated ischemia

To investigate EPI CdM-mediated protection of cardiac endothelial cells, human cardiac endothelial cells were purchased from Lonza (Catalog # CC-2585 and CC-7030, Passage 1). Cell protection assays were performed under conditions of simulated ischemia (low glucose medium, 1% oxygen, for 24 or 48 hr). We first performed an MTS assay (Promega) which measures cell metabolism, a measure of cell viability (Figure 4A). Compared with incubation in MEM (vehicle control), EPI CdM generated from hEPDC of donors from 52 to 80 years of age all protected primary human cardiac endothelial cells from simulated ischemic injury for 24 hr (Figure 4A). To determine the effects of EPI CdM on cell survival, we quantified cell number (nuclei) by dye-binding of the nucleic acids (cyQuant assay, Molecular Probes, Invitrogen). The CyQuant assay confirmed cell protection by EPI CdM (Figure 4B).

HGF is a key vascular protective component of EPI CdM

To identify signaling pathways stimulated in cardiac endothelial cells following exposure to EPI CdM, we performed phospho-receptor tyrosine kinase (RTK) arrays with lysates from human coronary artery endothelial cells previously exposed to MEM or 1x EPI CdM. We observed that c-Met, VEGFR1, VEGFR2, and Tie2 (angiopoietin-1 receptor), but not FGFR1 were phosphorylated after EPI CdM exposure based on array signal relative to that of MEM (Figure 4C). By ELISA, unconcentrated (1x) EPI CdM from

several different human donors contained HGF (about 3 ng/ml) (Figure 4D), angiopoietin-1 (ANG1, between 446 pg/ml and 1.1 ng/ml), VEGFA (between 500 pg/ml and 2.5 ng/ml), and SDF-1 alpha (<150 pg/ml) (Supplemental Figure 4).

To examine the relative role of HGF and other angiogenic factors in protection conferred by EPI CdM, we performed pull-down assays with growth factor-specific antisera.

Compared with non-specific PD, HGF pull-down significantly reduced the protective effects of EPI CdM (Figure 4E, Supplemental Figure 5). Importantly, pull-down of the other angiogenic factors from EPI CdM did not decrease its ability to protect endothelial cells under hypoxic conditions (Figure 4E).

Protein complexes containing HGF and IgG are present in concentrated EPI CdM

In experiments designed to neutralize HGF in 30x EPI CdM using protein A Sepharose, we observed an unexpected decrease in the HGF concentration of EPI CdM when it was incubated with non-specific polyclonal IgG alone (typically used as a control for specific IgG). We have previously described the presence of left-over serum carrier proteins in CdM preparations, due to dynamic recycling of extracellular proteins by cellular vesicles.²² We performed experiments to determine whether HGF interacted with IgG from serum in EPI CdM to form protein complexes as a consequence of concentrating it *ex vivo*. To test this idea, we first concentrated EPI CdM to varying degrees (1x to 40x). To determine if IgG was interacting with HGF we added a standard amount of IgG (non-specific polyclonal IgG, 2 ug/ml) to the series of EPI CdM concentrations to use as bait for HGF and performed pull-down assays with Protein A Sepharose. As expected, we detected increasing amounts of HGF by ELISA when unmodified EPI CdM was

concentrated from 1x to 40x. Hypothetically, in the instance that IgG and HGF were interacting, we would observe a reduction in HGF level and detect HGF on the Protein A beads. To test our hypothesis we performed pull-down of HGF from increasing concentrations of EPI CdM using a non-specific IgG, and compared the levels of HGF with before and after pull-down at the various concentrations (Figure 5A). We then tested the co-precipitation of HGF with IgG from EPI CdM, by washing the beads thoroughly after pull-down and then incubating them in sodium deoxycholate. We performed ELISA on the deoxycholate-soluble fraction to determine if we could recover HGF from the beads. We were able to detect increasing levels of HGF after pull-down with IgG, corresponding to increasing EPI CdM concentration. We detected about 1.5 ng/ml HGF from beads with 30x CdM and a significantly greater amount from beads with 40x CdM (~2.8 ng/ml Figure 5B) (n=3). Although some interaction may have been present, at EPI CdM concentrations lower than 30x CdM we could not detect HGF from beads by ELISA. In order to visualize the direct interaction between HGF and IgG in solution, electrophoretic mobilities of human IgG (Sigma) and human HGF were compared in free and complexed states by native agarose (1.5%) gel electrophoresis using MES buffer (50 mM, pH 6.7). By staining with Coomassie Brilliant Blue dye, we were able to observe a band shift for the HGF when loaded as a complex with IgG compared to free HGF (Figure 5C).

HGF/IgG complexes provide enhanced vascular protection by activating RYK

As the above protein interactions were observed *in vitro*, we were interested to learn if they had any physiological benefit(s). To investigate whether HGF/IgG complexes might

protect vascular endothelial cells differently during tissue injury than would free HGF, we performed *ex vivo* cell protection assays under conditions designed to simulate ischemia (1% oxygen combined with nutrient deprivation). To ensure reproducibility across experiments, we first produced and purified several mg of soluble, human HGF from stable clones of HEK293 cells that were grown in 5% serum (Figure 6A). Importantly, the recombinant HGF produced by this method was 100% processed by factors contained in serum (e.g. HGF-activating factor) as indicated by complete cleavage of the 90 kDa HGF protein into its subunits under reducing conditions (Figure 6A). Activity of HGF was further confirmed by performing an ELISA to measure activated c-Met receptor after exposure of human cells (Supplemental Figure 6). For protection assays, 100 ng/ml of HGF was added to cells as HGF/IgG complexes (see detailed description in methods) or as free HGF combined with a concentration of IgG that matched that of the HGF/IgG complexes. We observed a significant increase in protection conferred by HGF/IgG complexes compared with uncomplexed HGF and IgG at individual, matched protein concentrations ($p \leq 0.01$, $n=4$; Figure 6B). Notably, uncomplexed HGF with IgG provided similar level of protection in culture when compared to free HGF alone. We therefore chose uncomplexed HGF and IgG mixture as appropriate control for HGF/IgG complexes in subsequent experiments.

To determine if HGF/IgG complexes mediated their protective effects via a receptor (or receptors) that differed from c-Met, we performed a second phospho-RTK array with lysates of human coronary artery endothelial cells that were incubated for 30 min with HGF/IgG complexes or with a matched concentration of HGF and IgG. On the array, the level of c-Met phosphorylation was comparable in both the treatment groups, suggesting

an additional mechanism for protection mediated by HGF/IgG complexes (Figure 6C). Of special interest, we observed phosphorylation of RYK (a.k.a. related to tyrosine kinase) after treatment with HGF/IgG complexes but not after treatment with free HGF and IgG (Figure 6C). RYK is an “orphan” receptor tyrosine kinase with no ligand yet identified that promotes its phosphorylation.^{23,24} We next performed a cell protection assay under conditions of simulated ischemia in the presence or absence of RYK neutralizing antibodies. Blocking RYK on coronary artery endothelial cells significantly decreased the protective effect of the HGF/IgG complexes (complexed HGF with anti- RYK 155.46% \pm 14.62 % of MEM, complexed HGF with non-specific control IgG, 223.46 % \pm 10.307 % of MEM, $p \leq 0.01$; Figure 6D, n=4), but not the protection conferred by uncomplexed HGF with IgG ($p=0.09$; Figure 6D, n=4).

HGF/IgG complexes localize to blood vessels and promote vascular protection after MI

To test whether treatment by HGF/IgG complexes was advantageous over uncomplexed HGF with IgG *in vivo*, we compared the relative level of vascular integrity after MI with reperfusion. We mixed HGF (10 μ g/ ml) with non-specific polyclonal rat IgG (carrier) either in a complexed form or uncomplexed form and compared effects to MEM infusion. We then administered HGF/IgG complexes or uncomplexed HGF with IgG (control) to 2 groups of rats at the time of reperfusion, 2 hours after LAD occlusion (steady injection of 1 ml treatment with a 30 gauge needle into LV lumen at the apical end of heart). By FITC-albumin assay, treatment with HGF/IgG complexes significantly improved vascular integrity at 24 hr after ischemia-reperfusion when compared with treatment by free HGF and IgG or MEM (MEM, 544.1% \pm 94.18 of sham, n=4; Complex, 370.2% \pm

102.86 of sham, n= 11; free HGF, 516.5%± 53.93 of sham, n=8; Complex vs. MEM, $p \leq 0.001$; MEM vs. free HGF, n.s.; Complex vs. free HGF, $p \leq 0.01$; Figure 6E).

Immunohistochemistry using an antibody targeting the N-terminal His-tag of our recombinant HGF demonstrated a perivascular localization of His (HGF) when administered as HGF/IgG complexes, but we were unable to detect His (HGF) in tissue sections of control animals that received free His (HGF) and IgG (Figure 6F). For rats treated with HGF/IgG complexes, His (HGF) was readily detectable in larger vessels (Figure 6F,F') and the microvasculature and the remaining capillary bed within the infarcted region (Figure 6F'').

Discussion

Based on their multipotent capacity for differentiation and paracrine activity, hEPDC are a promising cell type for cardiac regenerative medicine.^{10,15} However, safe and effective clinical use of hEPDC will require a more complete understanding of cytokines and growth factors secreted by hEPDC and their roles under homeostatic conditions and after cardiac injury. Here we identified HGF as a factor produced by hEPDC that protected cardiac microvascular endothelial cells during hypoxia/ischemia. When administered upon reperfusion after MI, concentrated human EPI CdM prevented vascular leak and promoted microvascular cell survival. Previous reports demonstrated that HGF increased myocyte survival after cardiac injury (ischemia/reperfusion)²⁶ and provided vascular protection after lung or brain injury.^{27,28}

We found that EPI CdM contained unique HGF/IgG complexes that promoted endothelial cell survival in a manner superior to “free” HGF. Furthermore, we determined

that recombinant human HGF and IgG interact in a predictable manner *in vitro*, and, could be reproducibly prepared as complexes for *in vivo* administration. Compared with free HGF, HGF/IgG complexes increased the preservation of vascular integrity after myocardial ischemia with reperfusion. Immunohistochemical data suggested that HGF/IgG complexes localized to blood vessels in a different fashion than did free HGF, both spatially and temporally; this may increase local concentration and/or retention time, thereby enhancing its protective effect(s). Exposure of vascular endothelial cells to either HGF/IgG complexes or free HGF promoted the phosphorylation of c-Met to the same extent, but only HGF/IgG complexes were able to induce phosphorylation of RYK. Furthermore, protection of endothelial cells by HGF/IgG complexes was substantially diminished when RYK was blocked by antisera.

At present, the intracellular signaling cascade downstream of RYK phosphorylation has yet to be determined, but several reports describe RYK as a modulator of Wnt ligands and Wnt signaling.²⁹⁻³² RYK has also been shown to associate with Ephrins, which have known roles in vascular permeability during developmental angiogenesis and after injury.^{33,34} In the case of reperfusion injury and endothelial cell protection, RYK signaling could promote intracellular signal transduction that directly increases cell survival. Notably, HGF/IgG appears to be the first “ligand” reported to induce RYK phosphorylation and may therefore provide a tool to help delineate signaling downstream of p-RYK.

In the clinic, patients with MI commonly undergo percutaneous coronary intervention (PCI) or thrombolytic treatment (e.g. tissue plasminogen activator, TPA)³⁵ to restore blood flow to occluded vessels. Unfortunately, re-canalization of macroscopic vessels

does not lead to improved microvascular perfusion in ~30% of cardiac patients.³⁶ This phenomenon is described as “no re-flow” or “low re-flow” and the extent of no re-flow is a major determinant of infarct expansion after MI.^{37,38} No re-flow may result from destruction of microscopic vessels, which we have termed “vascular rhexis” or from other factors such as microemboli, inflammation, release of toxic cellular metabolites, or oxidative stress that cause endothelial cell dysfunction and induce microvascular leaks.^{39,40} Our results indicate that HGF/IgG treatment protects against reperfusion injury after MI as opposed to stimulation of angiogenesis, which is a process that requires several days. Further administration of HGF/IgG complexes may provide additional protection or induce angiogenesis, but this remains to be tested.

By simultaneously signaling through c-Met and RYK, HGF/IgG complexes have potential as a new therapeutic to improve vascular integrity. Infusion of HGF/IgG complexes may also reduce vascular rhexis and the incidence of no/low reflow after MI. In addition to cardiac ischemia/reperfusion injury, many other forms of tissue injury or disease that involve damage to vascular endothelium, such as stroke and peripheral artery disease, may also benefit from treatment with HGF/IgG complexes.

Acknowledgments

This work was supported in part by NIH grants HL077570 and HL085210 (to J.L.S.). We thank staff in the UVM Microscopy and Imaging Core and Matthew D. LeComte, Ph.D., for assistance with obtaining confocal images. Special thanks to A.K.M Tarikuz Zaman, M.D., for helpful discussion.

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Supplementary Material

Isolation of Adult Human Epicardial Progenitor Cells and EMT into Precursor Cells

Right atrial appendages were obtained from consenting cardiac bypass patients in a protocol that was approved by the IRB of the University of Vermont. The appendages were transferred from the hospital to the UVM Stem Cell Core on ice in 50 ml conical tubes containing explant medium: Alpha MEM (Invitrogen, Carlsbad, CA), 10% FCS (Atlanta Biologicals, Lawrenceville, GA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Mediatech Inc., Hendron, VA).

Method #1 In a cell culture hood, appendages were immediately rinsed in 1X PBS and any extracardiac fat was manually removed with fine scissors. The remaining tissue was transferred to a 100 cm² dish) containing 1X PBS supplemented with 1 mg/mL collagenase/dispase (Roche Applied Science, Indianapolis, IN) in which it was minced into approximately 1 mm³ pieces with sterile scalpel blades. The dish was placed into a sterile 37°C humidified cell culture incubator for 1.5 hours, with shaking every 10 minutes. The resulting tissue digest was collected and centrifuged at 600 x g for 5 min. The pellet was resuspended and washed in 25 mls of explant medium and centrifuged again. The final pellet was resuspended in 20 mls of explant medium and the digested fragments were split between 2 uncoated 100 cm² dishes. After 2-3 days, the dishes were supplemented by the addition of 5 mls of explant medium and then left undisturbed to allow for the adherence of tissue fragments. After 5-7 days, when fibroblast outgrowth from the explants had almost reached confluence, the dishes were washed once by PBS and the explant medium was changed to a medium that favored stem/progenitor cell

growth: DMEM/F12 with 3% FCS and 20 ng/ml EGF, 10 ng/ml bFGF, 10 ng/ml LIF (all growth factors from Sigma, Saint Louis, MO), 1 x ITS plus (BD Biosciences, San Jose, CA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. After 2-3 days, areas of epicardial progenitor cells could be observed to be proliferating in between the fibroblasts. The progenitor cells were morphologically distinguishable from surrounding cell types, did not mix with surrounding cells, and formed floating spheroids and cell aggregates that resembled bunches of grapes as they divided upwards into the medium rather than horizontally. By shaking the dishes and washing once with calcium- and magnesium-free PBS, the floating progenitor cells were collected. The resulting cells (epicardial progenitor cells) were cultured in petri dishes (uncharged) in stem/progenitor growth medium for up to several weeks (in some cases up to 2 months). The epicardial nature of the cells was clear as all of the cells were epithelial and expressed Keratin proteins. Under these conditions, the epithelial cells continued to produce floating cells. To induce EMT, the epicardial cells were collected, centrifuged at 600 x g for 5 min, resuspended in Claycomb medium (SAFC Biosciences, Sigma) with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and transferred to new dishes. Following adherence to culture plastic, the majority of the progenitor-like cells underwent EMT within 3 days into precursor cells and expanded rapidly in the Claycomb medium with 10% FCS.

We examined whether long-term growth in the stem/progenitor cell medium (low serum with growth factors) and in uncoated petri dishes was necessary to generate the precursor cells at the next stage of culture. This was not the case as primary floating epithelial progenitor cells obtained directly from initial the feeder layer cultures could also undergo

EMT when plated onto typical positively-charged cell culture dishes and incubated in the Claycomb medium with 10% FCS.

Method #2 : Epicardial explants were cultured for up to 7 d in explant medium to allow outgrowth of epithelial cells. Upon switching to the adult stem/progenitor medium as above, the epithelial cells similarly became refractile and formed spheres and “bunches of grapes”. These progenitor-like cells also expressed Keratins and could be induced to undergo EMT into EPDCs in Claycomb medium containing 10% FCS (as above) (pictured in Supplementary Figure S1).

Characterization of Cell Surface Epitopes.

Pellets containing 0.5×10^6 to 1×10^6 cells were suspended in 0.5 ml PBS and were incubated for 30 min at 4°C with monoclonal mouse anti-human antibodies that were pre-titered for flow cytometry. All antibodies except those against CD105 and NG2 (Beckman Coulter, Miami, FL) were purchased from BD Biosciences Pharmingen (San Diego, CA). After labeling, the cells were washed twice with PBS and analyzed by closed-stream flow cytometry (LSR II, Becton Dickinson, Franklin Lakes, NJ).

Immunoblotting.

The soluble fraction from above was diluted 1:1 in protein lysis buffer (20 mM Tris, pH 7.4; 1 mM EDTA; 10 mM Sodium Chloride; 0.5% v/v Triton X-100) containing protease inhibitor (Complete mini # 04693116001, Roche, Basel, Switzerland) and phosphatase inhibitor (P0044-1ML, Sigma). Protein concentration was determined with a commercial Lowry assay (BioRad DC Protein Assay). For each sample, 10 µg of protein was loaded on a 4-12% Bis-Tris gel (Invitrogen). Proteins were transferred to a PVDF membrane and blocked in 5% non-fat dry milk in TPBS for 1 hour at room temperature. Primary

antibodies were diluted in 5% non-fat dry milk and incubated with membranes overnight at 4 °C: phosphoVE-Cadherin (44-1144G pTyr658, Invitrogen; 1:500), α -smooth muscle actin (A5228 clone 1A4, Sigma Aldrich; 1:1000), and GAPDH (Clone 6C5, Chemicon, Millipore; 1:1000). Membranes were washed in TPBS for 10 min x 3 and incubated in HRP-conjugated secondary antibodies for 1 hr at room temperature. Membranes were washed for 15 min x 3 and signals were enhanced by chemiluminiscence (Perkin Elmer) prior to exposure on X-ray film (Kodak)

Immunohistochemistry and Immunocytochemistry assays.

Cells cultured in chamber slides were washed once with PBS and fixed by 10 min incubation in 4% paraformaldehyde in PBS. Following several PBS washes, the chamber slides were blocked for 1 hr with 5% goat serum with 0.4% Triton-X 100 in PBS.

Primary antibodies were diluted into blocking solution, placed on the cells, and incubated overnight at 4°C. The following primary antibodies were used: Smooth muscle myosin heavy chain, (SMMS-1, Dako, Carpinteria, CA 1:100), Von Willebrand Factor (F8/86, Dako 1:50), Fibroblast (prolyl hydroxylase) (clone 5B5, Dako 1:100), GATA 4 (G-4, Santa Cruz Biotechnology, Santa Cruz, CA 1:100) Cytokeratin (C2562, Sigma 1:100), Epicardin (TCF21, ab32981, Abcam; 1:100), Vimentin (E2944, Spring Bioscience, Freemont, CA 1:200) WT1 (C-19, Santa Cruz Biotechnology 1:100), RALDH2, (Bioss, bs-3676R 1:200). Following 3 x PBS washes, secondary antisera was applied in blocking buffer and incubated for 1 hr at RT. After 3 more washes, slides were mounted (Vectashield with DAPI, Vector laboratories, Burlingame, CA), cover slips were applied, and specimens were viewed with an epifluorescence microscope (Leica DM6000B with DFC350 FX camera). Immunohistochemistry was performed as described previously

(17). Hearts were isolated and fixed overnight in 4% paraformaldehyde. They were then equilibrated in 15 and 30 % sucrose consecutively (24 hr in each) followed by mounting in OCT. Hearts were sectioned in 10 μ m serial sections, apex to base. Fixed slides were heated on a heated block for 20 minutes at 52°C and washed 2 x 5 minutes in PBS. Slides were blocked in goat serum (5% goat serum, 0.05% Triton X100 in PBS) for 1 hour at room temperature followed by incubation in primary antibody overnight; mouse anti rat CD31 (1:50, BD Bioscience). The following day, slides were washed 2x5 minutes in PBS, and incubated in Alexa 594 conjugated goat anti mouse antibody. Slides were treated with 20 μ g/ml Proteinase K in PBS for 15 minutes at room temperature and washed thoroughly with PBS 2 x 5 minutes. Slides were incubated in equilibration buffer for 1 minute at room temperature followed by incubation in working enzyme solution with substrate for 1 hour at 37°C. They were then washed in stop/wash buffer for 10 minutes at room temperature followed by mounting with DAPI and mounting medium. RT-PCR.

Total RNA was isolated from cell pellets with a commercial kit (RNAqueous, Ambion, Austin, TX). To avoid the possibility of contaminating DNA, the total RNA samples were treated with DNase prior to reverse transcription (TURBO DNase, Ambion).

Reverse transcription was performed with Superscript III (Invitrogen) in the presence of RNase inhibitor (RNaseOUT, Invitrogen). PCR was carried out with an Eppendorf Master Cycler EP thermal cycler. Control RT-PCR reactions included template samples in which the reverse transcriptase was omitted from the single strand synthesis reaction. Primer sequences and annealing temperatures are listed below. Target sequences were denatured at 94°C (2 min) followed by 30 amplification cycles of 94°C (30 s), anneal

temp (30 s), and 72°C (45 s). The last PCR step extended the products at 72 C for 1 min. RT-PCR products were analyzed on 1% agarose gels. The following PCR primers were used: SNAIL, Forward 5' TTT ACC TTC CAG CAG CCC TA 3', Reverse 5' CCC ACT GTC CTC ATC TGA CA 3'; SLUG, Forward 5' GAG CAT ACA GCC CCA TCA CT 3' Reverse 5' GGG TCT GAA AGC TTG GAC TG 3'; TWIST, Forward 5' GTC CGC AGT CTT ACG AGG AG 3', Reverse 5' TGG AGG ACC TGG TAG AGG AA 3'; SMAD1, Forward 5' CTA CCC TCA CTC TCC CAC CA 3', Reverse 5' GCA CCA GTG TTT TGG TTC CT 3'; GATA5 Forward 5' CAC AAG ATG AAT GGC GTC AA 3' Reverse 5' CTT CCG TGT CTG GAT GCT TT 3'; Wt1 Forward 5' CGG GGG TGA ATC TTG TCT AA 3' Reverse 5' CCT GGA CCA TCC CCT ATT TT 3'; Isl-1, Forward 5' GTA GAG ATG ACG GGC CTC AG 3', Reverse 5' TTT CCA AGG TGG CTG GTA AC 3'; Tbx18 Forward 5' GGG GAG ACT TGG ATG AGA CA 3', Reverse 5' AGC AAG AGG AGC CAG ACA AA 3'; Tbx5 Forward 5' ACG TGC TCA GTT TTG CCT CT 3', Reverse 5' CAG TTT TGT GTT GGC ATT GG 3' Smooth muscle alpha actin Forward 5' GAA GAG GAC AGC ACT GCC T 3' Reverse 5' CTG ATA GGA CAT TGT TAG CAT A 3' vWF Forward 5' AAG AAC CGA AGT CCC AGG AGA AAG G 3', Reverse 5' AGA TTT CAG AGG CGT TCT AAA ACT CA 3' GATA4 Forward 5' GAC GGG TCA CTA TCT GTG CAA C 3', Reverse 5' AGA CAT CGC ACT GAC TGA GAA C 3'; Mef2C Forward 5' CTG GGA AAC CCC AAC CTA TT 3', Reverse 5' GCT GCC TGG TGG AAT AAG AA 3' Myocardin Forward 5' GGA CTG CTC TGG CAA CCC AGT GC 3', Reverse 5' CAT CTG ACT CCG GGT CAT TTG C 3' GAPDH Forward 5' GCT GAG TAC GTC GTG GAG T 3', Reverse 5' CAC CAC TGA CAC GTT GGC A 3'.

Pull down of HGF from EPI CdM.

Monoclonal goat anti-human HGF (R & D Systems) was incubated with 30x EPI CdM overnight at 4 °C on a rocking platform. The following day, 1 ml of EPI CdM was incubated with 250 µl of Streptavidin agarose (GE Healthcare) for 2 hr at 4 °C on a rotating platform. The agarose beads were washed twice thoroughly with PBS to remove traces of ethanol from the stock preparation. After incubation the beads with EPI CdM were centrifuged at 4 °C for 30 minutes at 800 x g to collect the “pull down” fraction that had separated with the pelleted beads. Sandwich ELISA (R & D Systems) was performed on EPI CdM after pull down to confirm depletion of HGF.

Recombinant human HGF

A recombinant human HGF (HGF) expression plasmid was constructed by cloning a human HGF cDNA from plasmid pBabe-puroHGF (B.Weinberg Lab, Addgene,# 10901) [29] into vector pIRES-puro3 (Clontech), with the addition of 6xHis Tag to the C-terminus. The plasmid was introduced into HEK293 cells by transfection (Lipofectamine 2000, Invitrogen) with puromycin selection of stable clones. For production of recombinant HGF, cells were grown in 150 mm² dishes in DMEM containing 5% FBS with medium changes every 3-4 days. For purification, medium containing secreted and processed HGF was clarified by 3 min centrifugation (2934 g) and ultrafiltration. Heparin-sepharose affinity chromatography (GE Healthcare) was used as 1 step of purification. Heparin-bound factors were eluted stepwise with 1 M NaCl, diluted, and applied onto Ni-NTA Resin (Novagen). HGF was eluted with 250 mM of imidazole (~95% purity of hHGF by SDS-PAGE).

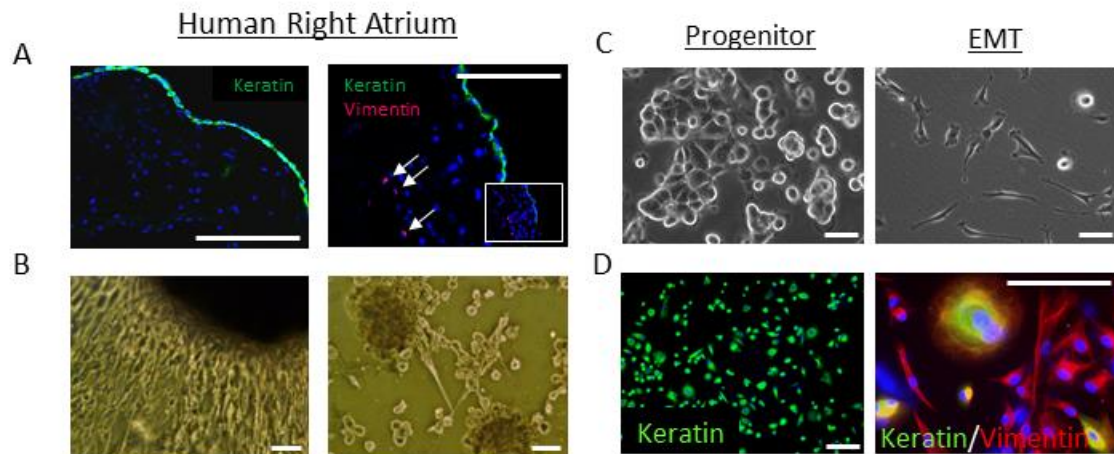


Figure 5- 1. Isolation of adult human epicardial progenitor-like cells and EMT into precursor cells

(A) Immunohistochemistry of a section of right atrial appendage from a human donor showing a single layer of keratin-positive epicardial cells (Right). Epicardial cells are Keratin-positive and not Vimentin-positive (mesenchymal marker). Arrows point to vimentin-positive cells. Inset: Note absence of vimentin staining in the epicardial layer (n=4 donors). (B) Explant culture of right atrial appendage to generate feeder layer (Left); Formation of floating spheroids and bunches of cells following switch to medium that favors the growth of stem/progenitor cells (right). (C) Maintenance of epicardial progenitor-like cells with epithelial phenotype on uncoated dishes (left), followed by EMT at 3 days after incubation of epicardial progenitor cells in medium containing 10% FBS (Right). (D) Epicardial progenitor-like cells express keratins (epithelial intermediate filament proteins) (Left) while precursors derived by EMT express vimentin (red) but not keratin (green) (Right) (n=5-7 donors). Scale bar = 100 μ M.

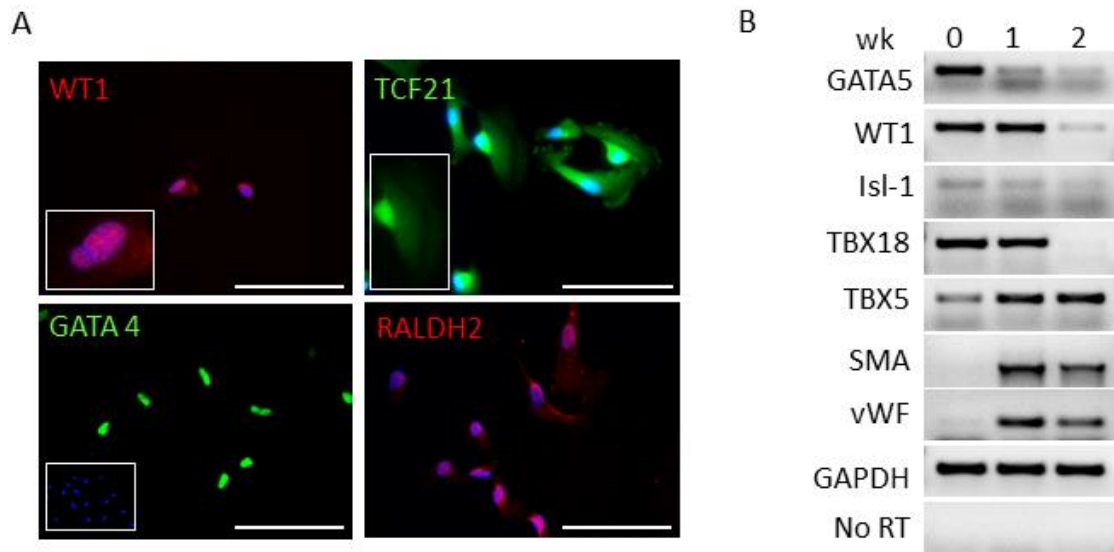


Figure 5- 2. Characterization of epicardial and precursor markers of the isolated human cells

Immunocytochemistry for epicardial transcription factors: WT1 (Top Left), TCF21 (Top Right), GATA4 (Bottom Left) and RALDH2 (Bottom Right) (n=5-7 donors). (B) Several transcription factors associated with cardiac development are downregulated in epicardial progenitor-like cells during EMT into precursor cells, while others such as smooth muscle actin (SMA) and vWF are upregulated. Left lane shows amplification from RNA of progenitor-like cells “EPI” pictured in (1C). Middle and right lanes show amplification of RNA from cells after EMT and 1 or 2 weeks of culture (n=3 donors). Note: The lower fuzzy bands in the gel data for GATA5, Isl-1, and Tbx5 are primer dimers. Scale bar = 100 μ M.

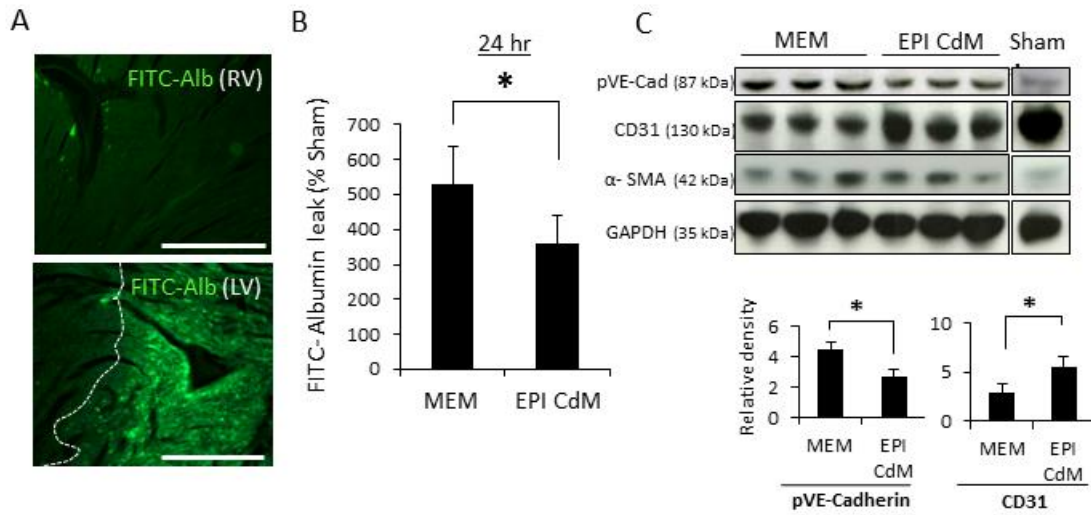


Figure 5- 3. EPI CdM treatment improves vascular integrity after myocardial ischemia with reperfusion in rats

(A) Representative immunofluorescence images showing the extent of FITC–albumin extravasation from an uninjured blood vessel in the right ventricle (RV, Top) and from an injured LV vessel (Bottom). Dashed white line indicates infarct border at 24 hr after reperfusion. We confirmed a similar staining pattern in hearts of 3 different animals (n=3). (B) Treatment with 30x EPI CdM significantly decreased extravasation of FITC-Albumin in the LV wall of rats, 24 hr after reperfusion (n = 5 animals per group). In each group, treatment animals are calculated as % of sham. (C) Western blot image of 3 representative animals per group showing lower levels of phospho-VE-Cadherin (pY⁶⁵⁸) and higher levels of CD31 in heart homogenates from EPI CdM-treated rats compared with MEM-treated rats. Note: α -SMA levels were similar in both treatment groups. (Bottom) Quantification of Western blot bands. The levels of phospho-VE- Cadherin were higher in MEM (control) vs EPI CdM (n = 3) and CD31 levels were higher in heart homogenates of EPI CdM-treated rats compared with MEM-treated rats (n = 3). GM: Growth Medium; Con CdM: Control (EPI) CdM; PD: Pull down; Con IgG: Control IgG. Student's t-Test: * p \leq 0.05. Scale bar = 100 μ M.

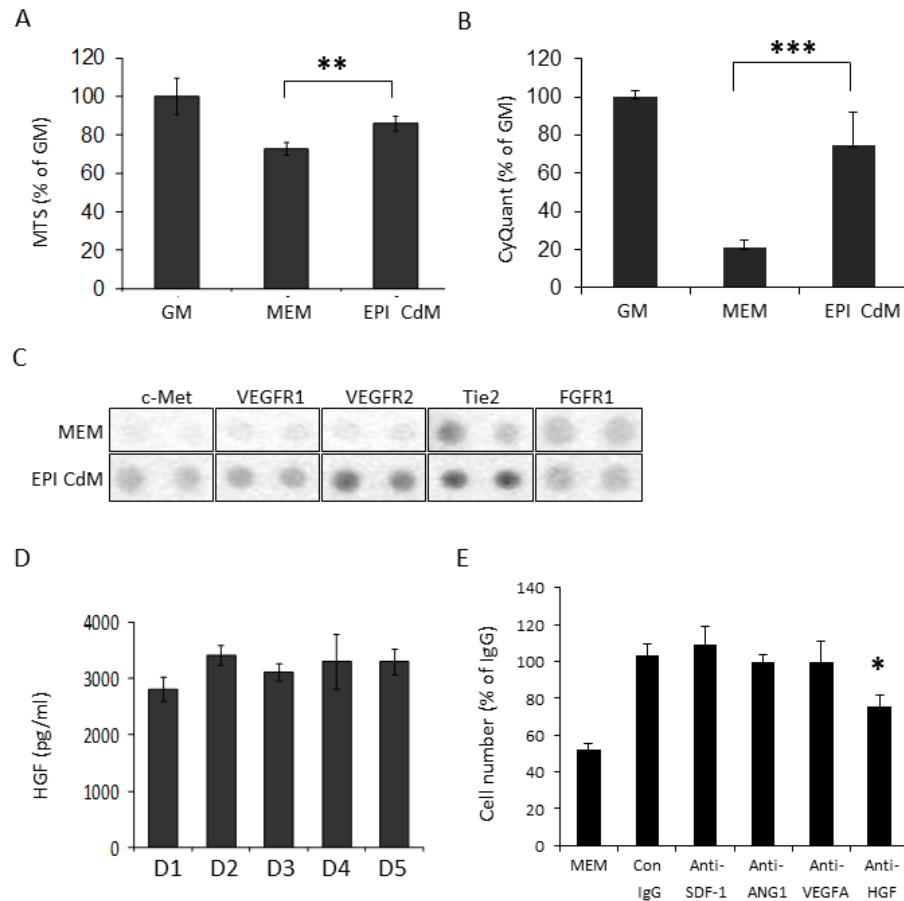


Figure 5- 4. HGF is a key vaso-protective factor in EPI CdM

(A) EPI CdM protected cultured primary human coronary artery endothelial cells for 48 hrs under simulated ischemia, as measured by MTS assay ($n = 5$ donors). Data are mean \pm S.D. (B) EPI CdM protected cultured human microvascular endothelial cells for 48 hr under simulated ischemia, as measured by CyQuant assay ($n = 3$ donors). Data are mean \pm S.D. In both experiments, survival of cells in growth medium (GM) was considered as 100%. (C) Receptor tyrosine kinase (RTK) array demonstrates 30 min exposure to 1x EPI CdM induces phosphorylation of multiple growth factor receptors of coronary artery endothelial cells compared with MEM treatment ($n=2$). (D) HGF ELISA data are for 5 different EPI CdM donors (D1-D5). Measurements are in duplicate. (E) Pull down (PD) of HGF from EPI CdM with an HGF-specific antibody (2 μ g/ml) decreased EPI CdM-mediated protection of microvascular endothelial cells during simulated ischemia (48 hr) compared to PD with a non-specific IgG antibody ($*p \leq 0.05$ Control IgG vs Anti-HGF). In contrast, PD of SDF-1, ANG1 or VEGFA did not alter protection of microvascular endothelial cells (all antibodies used at 2 μ g/ml) ($n=3$). Cell numbers were determined by CyQuant assay (dye binding of nuclei acids). Data are mean \pm S.D. One way ANOVA for A,B,E: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. Scale bar = 100 μ M.

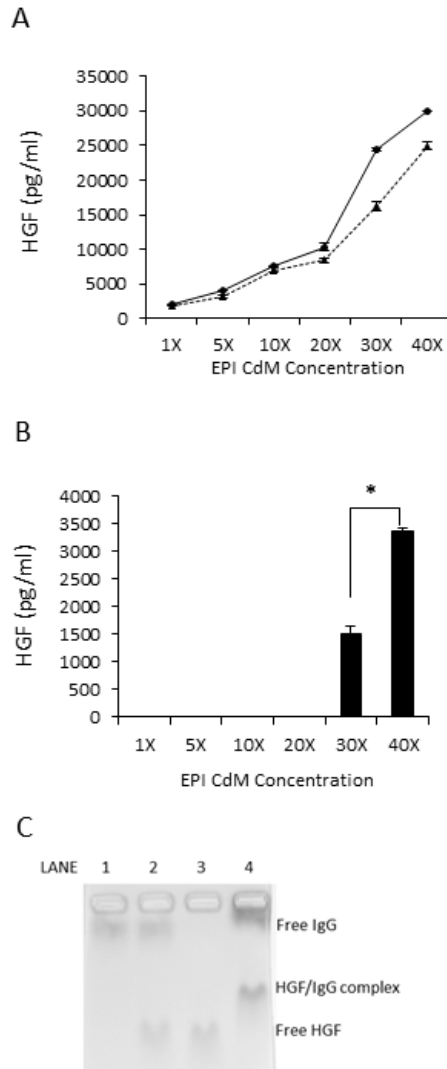


Figure 5- 5. Concentrated EPI CdM contains HGF/IgG protein complexes

(A) Loss of HGF from EPI CdM corresponded to increasing concentration of EPI CdM. Solid line= EPI CdM, dashed line= EPI CdM + non-specific polyclonal IgG (3 EPI CdM donors, n=3 each). (B) Quantification of HGF after dissociation from Sepharose beads with deoxycholate (3 EPI CdM donors, n=3 each). (C) EMSA (electrophoretic mobility shift assay) demonstrating the change in mobility of HGF when complexed with IgG. Human IgG (lane 1) migrates towards the anode at a much slower rate at pH 6.7 compared with human HGF (lane 3). A simple mixture of the 2 proteins (free) did not affect their individual mobilities (lane 2). However, HGF and IgG complexes formed by concentration resulted in a band-shift for both proteins as a result of altered mobility (lane 4) (n=3). Student's t-Test: * $p \leq 0.05$.

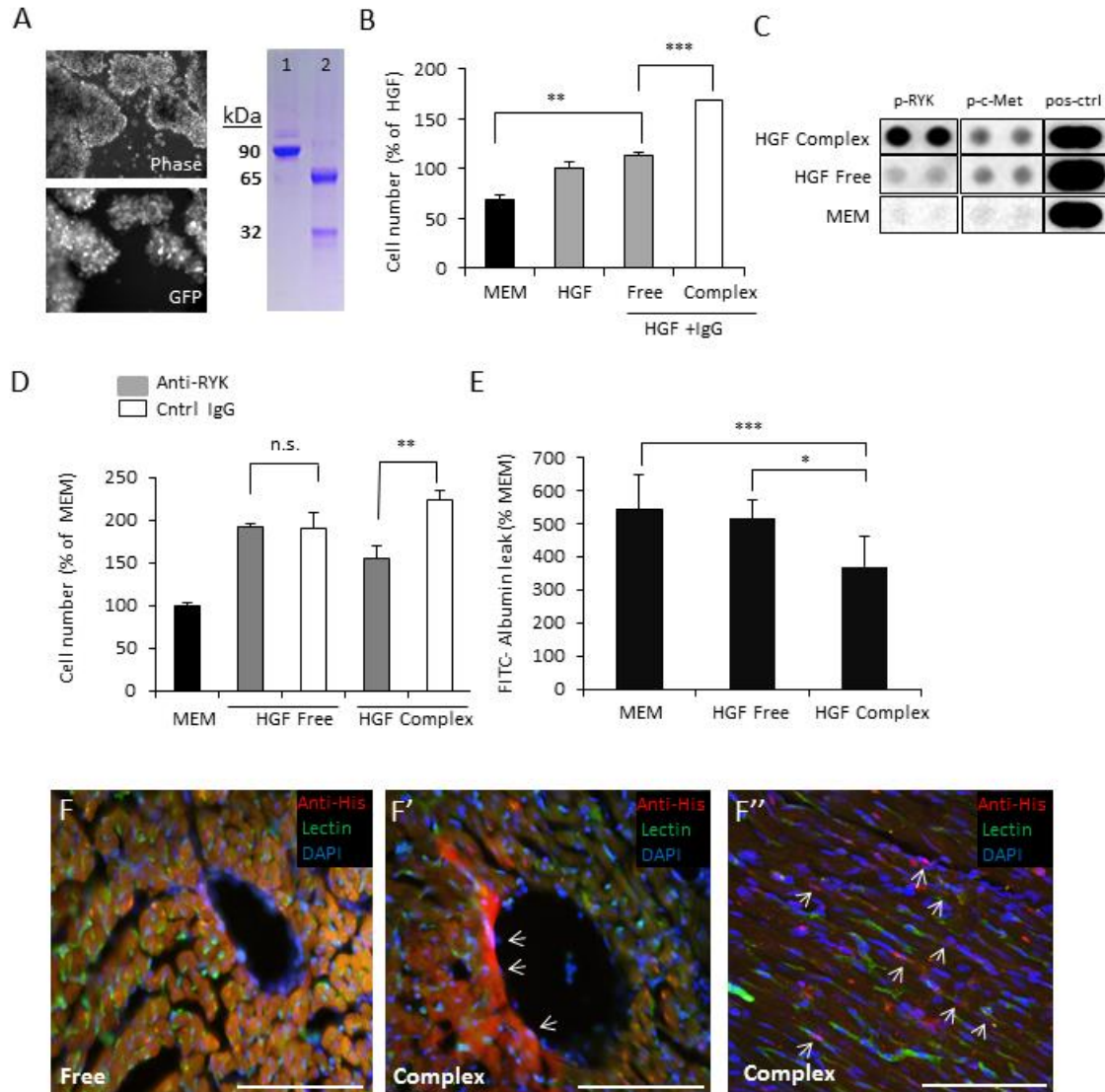


Figure 5- 6. HGF/IgG complexes enhance vascular protection by interacting with RYK

(A) Stable producer cell lines and purification of bioactive recombinant human HGF. (Left, Top) Phase contrast images of HEK 293 cells with genomic integration of pIRES-puro vector expressing human HGF-His tag-F2A-GFP. (Left, Bottom) Epifluorescence image showing GFP expression (FITC channel). (Right) Gel electrophoresis illustrating purification of HGF. Lane 1, Electrophoresis of eluted material under non-reducing conditions demonstrates purity and molecular weight of single chain pro-HGF (90 kDa). Gel stained with Coomassie Brilliant Blue. Lane 2, Purified protein run under reducing conditions (beta-mercaptoethanol) demonstrates the active heterodimer of human HGF (see bands at 65 kDa and 32 kDa) (n=4). (B) CyQuant assay demonstrates enhanced protective effects of HGF/IgG complexes when compared with free HGF (alone) or free HGF with IgG (Free, unconcentrated factors). For greater detail, please refer to Methods. Cell number was assayed after 48 hours of simulated ischemia (MEM, 68.52 ± 4.73 of HGF; free HGF alone, $100 \pm 7.27\%$; free HGF with IgG [Free], $113.65 \pm 3.117.27\%$ of HGF; HGF

with IgG (Complex), $169.034 \pm 4.004\%$ of HGF (n=4 for all). Free HGF with IgG (Free) conferred protection ($p \leq 0.01$, vs. MEM). However, HGF/IgG complexes (Complex) protected better than did free HGF with IgG (Free) ($p \leq 0.001$). (C) Treatment of coronary endothelial cells with HGF/IgG complexes (Complex, Top), free HGF and IgG (Free, Middle) or MEM (Bottom) affects the level of phosphorylation for RYK, but not c-Met. Positive control signal (pos-ctrl) has been included for each membrane to compare for normalization (n=2). (D) Blocking RYK reduced protection conferred by HGF/IgG complexes ($p \leq 0.01$, n=4) but not protection by free HGF with IgG ($p=.09$, n=4). Note: Coronary artery endothelial cells were incubated under simulated ischemia for 24 hr. (E) Intra-arterial treatment with HGF/IgG complexes significantly decreased FITC-albumin extravasation in the LV wall of rats at 24 hr after reperfusion (MEM, n = 4; free HGF with IgG [Free], n=8; HGF/IgG complexes [Complex], n=11; $p \leq 0.01$). (F,F') Epifluorescent image showing localization of His-HGF outside of blood vessels (by anti-His antibody) in animals treated with free HGF (F) and complex HGF (F'). HGF/IgG complexes were localized to large blood vessels (F, F') and the capillary network at the borders of infarcted regions (F''). We confirmed a similar staining pattern in hearts of 3 different animals. Note: The presence of HGF not localized to capillaries is likely indicative of leakage from damaged or dead blood vessels in the capillary bed. Data are mean \pm S.D. One way ANOVA for B,D,E: ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bar = 100 μ M.

Sex	Age	HTN	DM	CAD	MR	AS	LVEF
M	47	+	+	+	-	-	0.45
M	59	+	-	+	-	-	NI
M	57	-	-	+	-	-	NI
M	52	-	-	-	+	-	NI
M	57	-	-	+	-	-	NI
F	53	+	-	+	-	-	NI
M	80	+	-	-	-	+	NI
F	72	+	-	-	+	-	0.45
M	53	+	+	+	-	-	NI
M	78	-	-	+	-	-	NI
F	72	+	-	+	-	-	NI
F	60	+	-	+	-	-	NI

Table 2. Patient data for epicardial progenitor cell isolations

Abbreviations: HTN, hypertension; DM, diabetes mellitus; CAD, coronary artery disease; MR, mitral regurgitation; AS, aortic stenosis; LVEF, left ventricular ejection fraction.

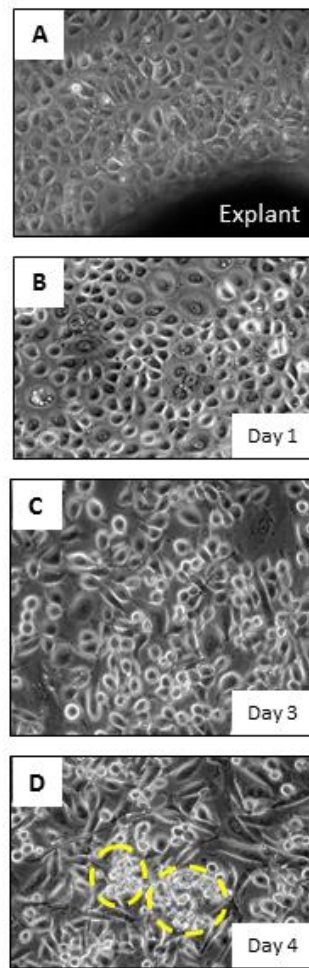
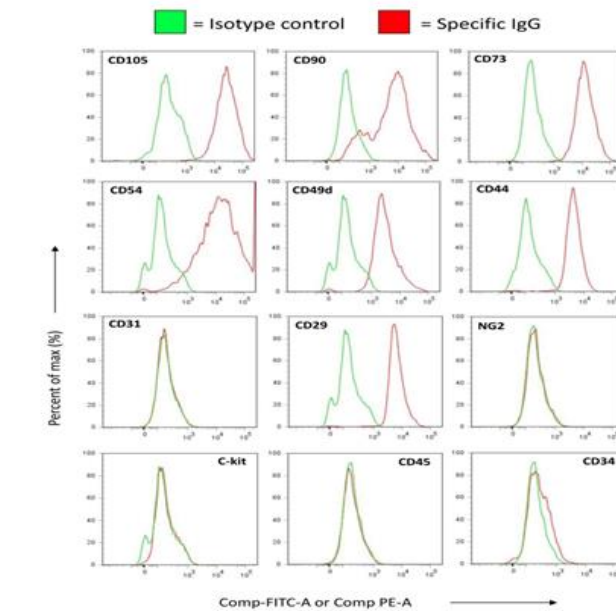


Figure 5- S 1. Time course of epicardial progenitor-like cell formation directly from human epicardial explant

Note that this time course pertains to isolation method #2 for EPDCs (see Methods). (a) Epicardial cell outgrowth from epicardial explant generated by dissecting surface epicardial cell layer from right atrial appendage removed during bypass surgery. Outgrowth occurs over 7 d. Note epithelial morphology of cell monolayer. (b) Epicardial cell monolayer 1 d after switching medium to adult stem/progenitor expansion medium (see Methods). (c) Epicardial cell monolayer 3 d after switching medium to adult stem/progenitor expansion medium. Note refractile cells beginning to round up. (d) Epicardial cell monolayer 4 d after switching medium to adult stem/progenitor expansion medium. Note the formation of “bunches of grapes” due to epicardial progenitor-like cells adhering to each other and growing upward in the culture dish (within yellow dashes). (e) As with isolation method #1, the bunches of progenitor cells pictured in (d) could be gathered and then transferred to a medium containing 10% FCS to induce EMT. Scales bars = 50 μ m



72 hrs

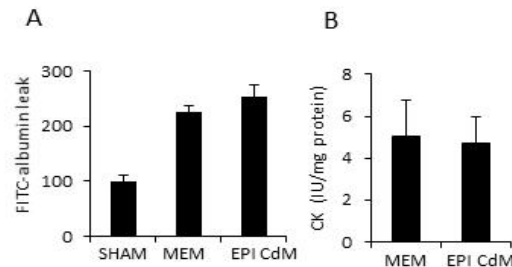


Figure 5- S 2. Cell surface phenotype of human EPDCs after EMT and cardiac function at 24 hrs

Many of the cells are positive for markers associated with human bone marrow-derived MSCs: CD105, CD90, CD73, CD54, CD49d, CD44, and CD29. Note that the expanding cell population is negative for CD45, CD34, and CD31 as well as c-kit and NG2. The green line represents the signal from cells stained with isotype control antisera and the red line represents the signal from cells from the same culture stained with specific antisera. (Bottom) A single, injected dose of EPI CdM does not protect against vascular leak at 72 hr after MI and treatment. (A) At 72 hr after reperfusion, there is no difference in FITC extravasation as measured by FITC-albumin ($n = 6$ animals per group, $p = 0.08$). (B) Myocyte survival in the LV at 24 hr was determined by assay of LV residual creatine kinase (CK) activity. There was no difference in the level of myocyte survival as indicated by residual creatine kinase activity between the MEM and EPI CdM treatment groups at 24 hr ($n=5$ animals per group, $p=0.74$).

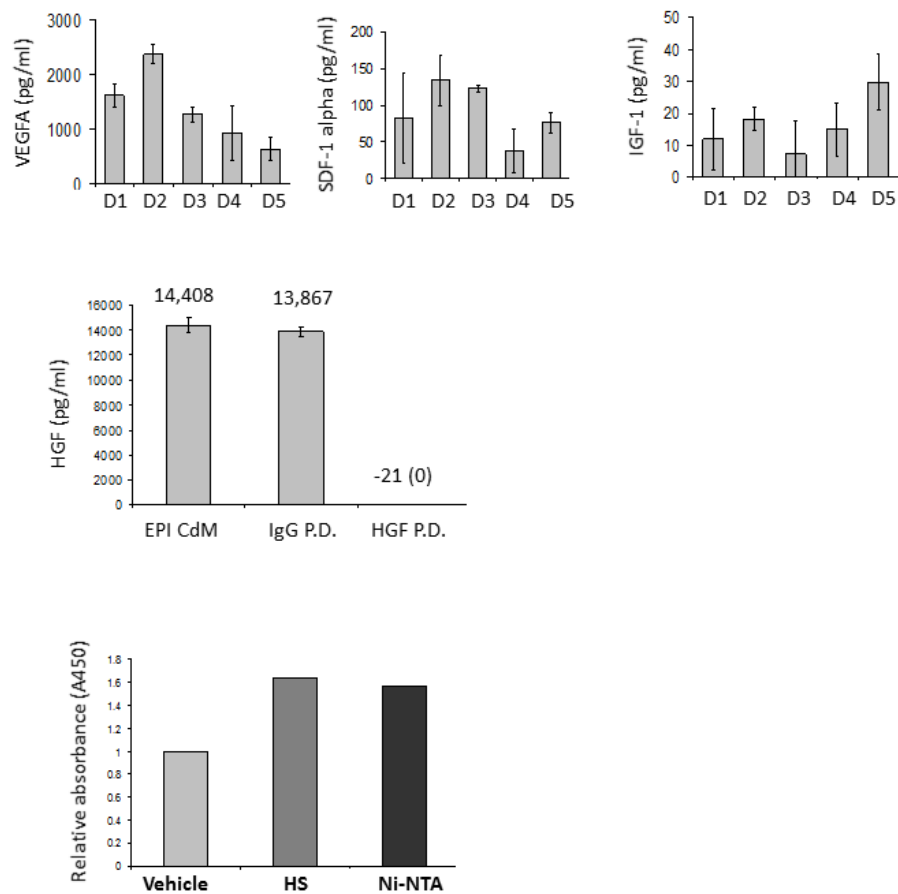


Figure 5- S 3. Additional data for proteins secreted by EPDCs

ELISA data for VEGFA and SDF-1 alpha. Data are shown for 5 different human donors that range in (Middle). Pull down of HGF from EPI CdM. Using HGF specific antibody, HGF can be completely depleted from EPI CdM as measured by ELISA. (Bottom). Activity of purified recombinant human HGF. ELISA data for human phospho-HGF R/c-Met (R and D Systems) shows activity of material eluted from Heparin-sepharose column (HS) and Ni-NTA column (Ni-NTA material diluted 5-fold). Cultured human epithelial cells were incubated 1 hr in vehicle or elutions and then lysed for ELISA.

CHAPTER VI. DISCUSSION

Concluding remarks

The goal of this dissertation was to advance the understanding of epicardial cell signaling and functions in the adult mammalian heart. I focused on evaluating the contribution of epicardial cells in cardiac regeneration during growth (exercise) and in response to injury. The studies were ultimately aimed at determining whether epicardial cells are useful for cardiac cell therapy and/or can serve as a source of beneficial paracrine factors for the treatment of myocardial ischemia/reperfusion. My dissertation research sought to define new methodologies for the study of epicardial cells both *in vivo* and *ex vivo*. I present data describing epicardial cell identification in mammals, and the isolation, characterization and long-term propagation of epicardial cells and their derivatives *ex vivo*. These findings are confirmed by data collected from multiple donor species. Current controversies and technical limitations for studying epicardial cells *in vivo* prompted us to develop a new mouse model that employed a Keratin18CreER^{T2} (K18 CreER^{T2}) mouse for performing gene screening and targeted gene knockout in epicardial cells. This model constitutes a significant advance over currently available adult transgenic mouse models where transgene expression relies on the initiation of an injury for the re-expression of embryonic Cre driver genes (Zhang et al., 2013).

Summary of interests and future directions

Screening mesenchymal stromal cell (MSCs) from the bone marrow niche for paracrine factors that support stem/progenitor cell survival and function, we identified a defined

combination of CTGF-D4 and Insulin that promoted robust cardiac stem cell grafts to the heart after MI. These 2 peptides also promoted the engraftment of transplanted primary EPDCs after MI/R into their original subepicardial niches, where they survived, proliferated and initiated migration to participate in repair and remodeling. Alternatively, my experiments with transplantation of primed epicardial cells directly into the myocardium, or into regions distal to the infarct, did not demonstrate a similar level of graft success. I therefore infer that the most successful engraftment of EPDCs likely occurs when they were transplanted back into their original *in vivo* sites. Notably, these are also sites which corresponds to sites where multiple tissue repair mitogens become concentrated after MI (Chen et al., 2012; Mehrabi et al., 2002; Tofukuji et al., 1998; Tomanek et al., 2006; Tomanek and Zheng, 2002). These observations indicate that there are cardiac “injury niches” within subepicardial regions that border tissue with infarction after MI. Employing subepicardial border zone cell delivery, we transplanted cultured EPDCs back into these injury niches, thereby exposing them to additional signals *in vivo* to support their survival, proliferation and migration. EPDC engraftment into subepicardial sites was superior to other methods of administration (Hamdi et al., 2009; Tano et al., 2014). This observation relates to a parallel clinical consideration regarding the site of injection or method of administration. While direct injection into the myocardium with infarction affects cell viability due to necrosis, and systemically injected cells face the risk of non-specific embolization to the lungs, my results with subepicardial injections suggest it may provide a promising strategy for clinical applications of EPDCs (Freyman et al., 2006; Malliaras et al., 2014; Mushtaq et al., 2014; Nakamuta et al., 2009; Scorsin et al., 1996; Sullivan et al., 2015). Importantly, the use of

animal models provided a means to perform open chest manipulations for cell administration, which, in humans, is performed only under certain instances. However, more recent methods for closed-chested manipulations that are clinically performed in other instances significantly increase the feasibility of this method of cell administration.

Optimizing protocols for engraftment helped us identify multiple interacting players in epicardial cell signaling such as LRP6, Sox9 and ETBR, with previously unrelated roles. Whereas these factors functionally integrated to form a novel signaling axis that controlled epicardial proliferation, EMT and migration, I do not rule out the existence of other factors with key roles in regulating these processes. For instance, my inhibitor screen also identified FGF to be critical for CTGF-D4 signaling on epicardial cells, which is consistent with observations by other groups (Masters and Riley, 2014; Zhou et al., 2011). Likewise, priming epicardial cells with a pharmacological Wnt pathway agonist “BIO” (Atilla-Gokcumen et al., 2006; Sato et al., 2004), augmented EPDC grafts that, in turn, provided a significant functional benefit, thereby implicating the classical Wnt pathway in controlling engraftment. Further studies will be needed to discern if these pathways function in a manner that is involved with or independent from the axis we describe.

During homeostasis and after MI, the epicardial cell layer provides a population of migratory progenitor cells (Gittenberger-de Groot et al., 2010; Weeke-Klimp et al., 2010; Wessels and Perez-Pomares, 2004), whose complete properties and functions have yet be uncovered. Importantly, the reliable targeted knockout model we generated with the K18CreER^{T2} mouse now permits specific cellular ablation of the epicardial cells in adults. This should help to determine whether they are required or dispensable for repair

after MI. To this end, I propose to compare developmental models of growth with adult models of hypertrophic growth to determine unifying features of epicardial fate decisions in the heart. Lineage-tracing using the K18CreER^{T2} reporter mouse model will be valuable to determine if EPDCs adopt different fates after EMT under different physiological circumstances, and the corresponding intracellular or extracellular molecular differences that control fate decisions (Braitsch et al., 2013; Lie-Venema et al., 2007; Munoz-Chapuli et al., 2002; Ruiz-Villalba et al., 2015; Smart et al., 2013). Based on gross histological observations in my experiments with exercised and lineage-traced animals, and extensive genetic inheritance studies from experts in the fields of cardiology and fibrosis (Galindo et al., 2009), I expect distinct cell fate outcomes in the different physiological models. These outcomes may depend on the physiological demands of growth versus repair and/or remodeling in cardiac tissue.

Among multiple functions of epicardial cells, one that has interested us and many other groups is their ability to secrete a range of growth factors that can be exploited for clinical use. By performing gene expression analysis on epicardial cells from human donors before and after EMT in culture, I noted gene transcription for various growth factors like CXCR4, VEGF-C, HGF, Endothelin-1 etc., after they underwent EMT. Intriguingly, I observed that exposure of epicardial cells to CTGF-D4 appeared to control the paracrine secretions of these cells. Specifically, CTGF-D4 stimulation of epicardial cells in culture increased their secretion of HGF, as measured by ELISA. I believe that the same LRP6/Sox9/ETB_R signaling axis that controls EMT and cell engraftment may also control the paracrine action of epicardial cells. This hypothesis is supported by computational analysis indicating that HGF has Sox9 binding elements in its promoter

and can potentially be regulated in a manner similar to what we reported for EDNRB. Gene array analysis of epicardial cells after EMT also suggested increased secretion of immune modulatory factors like IL-6, IL-21, TGF-beta, and other macrophage and monocyte attractants. More recently in my discussions with other groups, unpublished results from their work suggest that epicardial EMT could have significant roles in modulating immune infiltration after MI (Cantarini et al., 2013; Lau et al., 2011; McLarty et al., 2011; Vinci et al., 2013). Notably, preliminary observations from my transgenic mouse experiments with epicardial specific ETB_R knockout mice also indicate that a greater number of immune cells migrate into zones of MI after knockout. If these correlations prove to be true, our signaling axis might represent a rare example of a cell culture system that models the multiple effects controlled by epicardial cell functions *in vivo*. The process of EMT in adult epicardial cells requires additional investigations to better understand the signals that control epicardial cell fate and functions after injury. Furthermore, comparing cardiac function data between epicardial-specific knockout ETB_R and control animals after MI may provide valuable information regarding the requirement of EMT for functional improvement after injury.

Clinical perspective

The ability to detect and measure cell grafts at 1 month after transplantation in our studies provides a valuable opportunity to evaluate whether there exists a direct correlation between numbers of grafted cells and improved cardiac function. To better evaluate functional benefits in response to administration of cells or purified factors, I believe we need to include other measures of cardiac function in addition to echocardiography,

combined with systematic multiple dosing studies. This information will be important for defining the initial cell numbers for engraftment and effective dosage for pure factors, in a patient- specific manner.

For clinical application of epicardial cells, we need to increase their persistence *in vivo* after transplantation (Arnesen et al., 2007; Marelli et al., 1992; Ng, 2004; Penn, 2006; Stamm et al., 2012). One strategy could involve the phenotyping of donor EPDCs to create a cell bank. Alternatively, I propose considering the selection of cell lines derived from induced pluripotent (iPS) cells or clonal lines of EPDCs after genetic engineering, to capture their capacity for long-term proliferation. As a next step, it may be possible someday to genetically or chemically control the time course of epicardial migration *in vivo* and cell fate decisions for enhanced repair after transplantation. For instance, a few research groups have shown the ability to reprogram fibroblasts *in vivo* into myocytes using lentiviral injections after MI (Addis and Epstein, 2013; Fu et al., 2013; Lian et al., 2014; Sadahiro et al., 2015).

In order to evaluate the beneficial effects of epicardial-derived factors for clinical purposes, it is critical to optimize their preparation and administration regimen, in addition to analyzing their effects *in vivo*. As a first step, sensitive and reliable methods are required to detect whether signaling complexes of HGF/IgG exist in subjects, as my preparations were produced in an artificial system *in vitro*. Performing genetic or chemical modifications in epicardial cells to express reporters and tags at specific regions in the heart after injury should help to record their behavior and myriad roles in real-time. Such modifications may provide new experimental strategies for understanding paracrine

biology, while also possibly identifying means to amplify the desirable effects of paracrine factors *in vivo*.

Taken together, the studies herein provide unique insight into adult mammalian epicardial cell biology during homeostasis and after cardiac injury. Overall, these findings have potential to advance the use of epicardial cells and their derivatives for cardiac regenerative medicine.

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APPENDIX I. OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS THROUGH ALGINATE-GRAFT- POLY(ETHYLENE GLYCOL) MICROSPHERE-MEDIATED INTRACELLULAR GROWTH FACTOR DELIVERY

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Introduction

Osteoporosis is defined by a reduction in the quantity and quality of bone that results in skeletal fragility [1]. As people age, bone resorption rates become unbalanced and bone resorption dominates over bone formation, which leads to reduced bone mass and altered

bone architecture. Human bone marrow progenitor cells known as multipotent stromal cells or mesenchymal stem cells (MSCs) can differentiate into osteoblasts, chondrocytes and adipocytes and show promise for clinical bone repair [2]. A notable recent study with murine MSCs showed that intracellular as opposed to extracellular signaling of vascular endothelial growth factor A (VEGFA) had the ability to distinctly regulate MSC lineage commitment toward osteoblasts or adipocytes [3]. Liu et al. (2012) demonstrated that murine bone marrow stromal cells were more likely to differentiate into osteoblasts when MSC expressed VEGFA and possessed intracellular VEGFA, compared with no change in osteoblast differentiation following exposure of MSCs to extracellular VEGFA. The investigators proposed that VEGFA regulated differentiation through an intracrine mechanism unique to the intracellular form of VEGFA and different from the typical mechanism for secreted, extracellular VEGFA and signaling through cell surface receptors. For patients, the intracellular delivery of VEGFA into MSCs prior to cell therapy could be a potential approach to speed the repair of large or complex bone fractures or perhaps even to treat chronic skeletal diseases such as osteoporosis [4]. In this work, we developed and functionally tested a specialized drug delivery system designed to accomplish the controlled, intracellular delivery of VEGFA in MSCs.

Modern drug delivery systems are designed to maintain the structure and bioactivity of biomolecules and to release therapeutics in a controlled and predictable manner. Micro-encapsulation is one of the core technologies used in polymer drug delivery systems [5]. Polymeric particles or microspheres for controlled drug delivery applications are designed to provide uniform particle dimensions, shield the drug from the extracellular environment, and be biocompatible [6]. Alginate microspheres have

attracted much attention for the development of controlled- and sustained-release drug delivery systems for proteins [7], cytokines [8] and cells [9, 10]. Alginate is a naturally occurring polysaccharide extracted from brown seaweed that is generally regarded as non-toxic. The fabrication of alginate microspheres is favorable for drug delivery due to the relatively mild yet rapid gelation process that omits the use of harsh chemicals to ensure stability of encapsulated biomolecules [11-14]. However, the application of alginate microspheres has been limited due to their relatively large diameters (10 – 100 μm) and rapid drug-release rates (< 24 h) [15]. In addition, the anionic nature of alginate interferes with the encapsulation and release of charged molecules such as polyelectrolytes. To overcome these limitations, alginate microspheres have been refined by varying molecular weight and concentration, stirring conditions, degree of crosslinking, and chemical modification of the alginate polymer to achieve sustained drug release [16, 17].

Poly(ethylene glycol) (PEG) is the most widely applied synthetic polymer in the emerging field of biomaterials for drug delivery. Recently, alginate has been modified with PEG for the fabrication of microcapsule coatings [18], mucoadhesive polymers [19, 20], self-assembling nanospheres [21, 22] and hybrid microspheres [23, 24]. The ability of PEG to influence the pharmacokinetic properties of drugs and drug carriers has been used to modify many different pharmaceutical compounds and components [25]. Biocompatibility and stealth behavior make PEG an ideal material to avoid opsonization and subsequent elimination by the reticuloendothelial system [26]. In addition, PEG-modified products are less immunogenic and antigenic; hemolysis and aggregation of erythrocytes may also decrease, as can the risk of embolism. However, the lack of a cell

adhesion ligand limits its application in targeted drug delivery systems. To address this issue, we developed novel PEG-modified alginate copolymer microspheres with and without the surface conjugation of a bioactive cell adhesion ligand.

Proteins containing tri-peptide arginine-glycine-aspartate (RGD) attachment sites, along with the integrins serving as receptors for them, constitute a major recognition system for cell adhesion to the extracellular matrix (ECM) [27]. Consequently, researchers continue to utilize RGD in order to mimic cell adhesion proteins and bind to integrins [28], inhibit apoptosis, angiogenesis, and tumor formation [29], coat surfaces for use as biomaterials [30], and enhance drug delivery systems [31, 32], including microspheres [33]. In this study RGD was utilized as a model cell-recognition molecule to demonstrate the efficacy of immobilizing ligands onto microsphere surfaces. Our results demonstrate that the incorporation of an adhesion ligand onto the surface of alginate-graft-PEG (Alg-g-PEG) microspheres containing VEGFA provides a promising strategy to regulate osteoprogenitor cell differentiation and bone tissue homeostasis, in addition to providing efficacy for the use of surface ligands for cell-targeted therapeutic applications *in vivo*.

Materials and methods

Materials

Sodium alginate (MW = 65–75 kg/mol, 60-70% guluronic acid residues) was generously donated by FMC BioPolymer. Cysteine-L-arginyl-glycyl-L-aspartic acid (CRGD) was purchased from Genscript. Amine-poly(ethylene glycol)-thiol (NH₂-PEG-SH, MW = 1000 g/mol) and methyl-poly(ethylene glycol)-amine (mPEG-NH₂, Mw = 500 g/mol)

were purchased from Laysan Bio. N-ethyl-N'(3-dimethylaminopropyl) carbodiimide hydrochloric acid (EDC), N-hydroxysuccinimide (NHS), 2,2'-dithiodipyridine, methanol (MeOH, anhydrous, 99.8%), biology-grade mineral oil, Span 80, Tween 80, ethylenediaminetetraacetic acid (EDTA), dexamethasone, ascorbic acid, β -glycerol phosphate, hydrocortisone, isobutylmethylxanthine, indomethacin, deuterium oxide (D₂O), the In Vitro Toxicology Assay Kit (MTT-based), Hoechst 33342 and Alizarin red were purchased from Sigma-Aldrich. One molar hydrochloric acid (HCl) and 1 M sodium hydroxide were purchased from BDH ARISTAR®PLUS. Dichloromethane (DCM, 99.9%), sodium citrate, isopropanol, sodium chloride (NaCl), sodium acetate (NaAc), alpha-modified eagle medium (α -MEM, Hyclone), DyLight 550 Microscale Antibody Labeling Kit, and 20X phosphate buffered saline (PBS) were purchased from Fisher Scientific. Fetal bovine serum (FBS) was purchased from Atlanta Biologics and screened for a lot that best supported growth of human MSCs. Penicillin, streptomycin and Trypsin EDTA were purchased from Corning Cellgro. AdipoRed™ was purchased from Lonza Inc. Human VEGF DuoSet and human osteoprotegerin ELISA Kits were purchased from R&D Systems. Oligo (dT) and dNTP mix were purchased from Promega Inc. A Roche High Pure RNA Isolation Kit was purchased from Life Science. Polymerase chain reaction (PCR) primers were purchased from Integrated DNA Technologies.

Alg-g-PEG-S-S-Pyridine copolymer synthesis

First, NH₂-PEG-SH (0.1 mg, 0.1 mM) was dissolved in 5 mL of de-gassed acetate buffer (0.1 N sodium acetate adjusted to pH 4.6 with acetic acid, 0.3 M sodium chloride, and 1 mM EDTA) into which a solution of 2,2'-dithiodipyridine (88.124 mg, 0.4 mM)

dissolved in 10 mL of MeOH was added; the mixture was stirred at room temperature under a flowing N₂ atmosphere for 4 h. The MeOH was extracted three times with DCM and the sample was prepared for ¹H-NMR analysis [34].

Alginate was modified with the newly formed NH₂-PEG-S-S-pyridine using EDC and NHS chemistry. The COOH:EDC:NHS molar ratio remained consistent at 1:8:3.2 during the carbodiimide reactions, where COOH refers to the moles of alginate carboxyl groups. A 1% (w/v) alginate solution was adjusted to pH 5 with 1 N HCl. EDC was then added followed by NHS and the solution was mixed at room temperature for 30 min. NH₂-PEG-S-S-pyridine and mPEG-NH₂ (the control PEG graft chain) were added to separate alginate solutions, respectively; the target degrees of PEG modification of the alginate was 10 %. After 12 h of reacting at room temperature, the various alginate and PEG polymer solutions were placed into dialysis cassettes (MWCO 20 kDa, Pierce Biotechnology) and dialyzed against deionized (DI) water for 4 d; dialysis solution was changed every 12 h. The Alg-g-PEG-S-S-pyridine copolymer product was lyophilized and the powder was stored in a desiccator until use. PEG retention post-fabrication was verified using ¹³C-NMR (Bruker AVANCE III 500 MHz high-field NMR spectrometer) in D₂O [34]. As a control, Alg-g-PEG copolymers were synthesized with this approach using mPEG-NH₂. The polymer was dissolved in D₂O and the result was verified via ¹H-NMR (Bruker AVANCE III 500 MHz high-field NMR spectrometer).

Microsphere fabrication

To fabricate microspheres containing VEGFA, Alg-g-PEG-S-S-pyridine was dissolved in phosphate buffered saline (PBS) with pH pre-adjusted to 5.0 with 1 N HCl. Non-modified alginate (Alg) and Alg-g-PEG microspheres were fabricated using a 1% (w/v)

polymer solution. VEGFA was added to the copolymer solution at a ratio of 106:1 (copolymer:VEGFA). Microspheres without VEGFA were fabricated as blank controls. One mL of polymer/VEGFA solution was slowly added to 6.72 mL of biological-grade mineral oil containing 5% (v/v) Span 80 while mixing at 1200 rpm for 5 min at room temperature. Next, 400 μ L of 30% (v/v) Tween 80 was added and the emulsion was mixed for an additional 5 min. Then, 5 mL of 1 M calcium chloride (CaCl_2) solution was added slowly. After 30 min of mixing, 3 mL of isopropanol was added to the emulsion and allowed to mix for 5 min, then was centrifuged at 400 rpm for 5 min to precipitate microspheres. The microspheres were washed sequentially with isopropanol (x2) and DI water (x2), respectively, and centrifuged after each wash. The Alg-g-PEG-S-S-pyridine microspheres were formed in a similar fashion, however, the DI water wash was replaced with a 1% (w/v) CRGD solution (x2); the corresponding microspheres are here-after identified as Alg-g-RGD. Microspheres were flash frozen in liquid N₂.

After lyophilization Alg, Alg-g-PEG and Alg-g-RGD microspheres were characterized by scanning electronic microscopy (SEM, JEOL 600; samples were sputter coated with 45 nm of gold). SEM micrographs of various magnifications were used to quantify microsphere diameters; images were analyzed using ImageJ (3 images per group).

Cytotoxicity assay

Human MSCs were isolated from bone marrow aspirates with an IRB-approved protocol. Human MSCs (passage 7) were seeded in 24-well tissue culture polystyrene (TCPS) plates at a density of 20,000 cells/well in 500 μ L/well of standard MSC growth medium (α -MEM, 10% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin) and allowed to

adhere for 24 h. Cells were incubated in the presence of Alg, Alg-g-PEG, and Alg-g-RGD microspheres encapsulating VEGFA (n = 3 per group) at concentrations of 10, 50, 100, 500 $\mu\text{g/mL}$ at 37°C and 5% CO₂. After 24 h of incubation, medium containing the microspheres was removed, and cells were rinsed two times in sterile PBS then analyzed using a MTT-based In Vitro Toxicology Assay Kit following the manufacturer's protocol. The optical density was measured at 570 nm using a BioTek plate reader. Background absorbance at 690 nm was subtracted from the measured absorbance. Absorbance values for the experimental and control samples were normalized to non-modified TCPS controls [35].

DyLight 550 labeling of human VEGFA

Recombinant human VEGFA was cloned, expressed in HEK 293 cells, and purified in the Spees laboratory [36]. 1 mg/mL of purified VEGFA in PBS was used for labeling. The DyLight Microscale Antibody Labeling Kit was used according to the manufacturer's instructions. Briefly, 100 μL of 1 mg/mL protein was mixed with a commercial vial of DyLight 550 reagent and incubated for 60 min in the dark after gentle vortexing and a quick centrifugation step to mix the sample and dye. Spin columns provided by the kit were then placed into microfuge collection tubes and used for purification of protein from unbound dye. The labeling solution was mixed with purification resin and centrifuged in the spin column to purify labeled protein. The dye:protein ratio was determined based on methods described in the labeling kit.

VEGFA encapsulation and release

A known amount of lyophilized VEGFA-loaded Alg, Alg-g-PEG and Alg-g-RGD microspheres was dissolved by immersion in 3% (w/v) sodium citrate solution to dissolve

the microspheres by displacing calcium ions [37]. The VEGFA concentration was measured with a DuoSet ELISA Development Kit. Briefly, standard series and sample solutions were added to a culture plate (100 μ L/well) pre-coated with capture antibody after washing in mild detergent (0.05% Tween 20 in PBS). After 2 h of blocking and incubation at room temperature, the biotin-conjugated detection antibody was added and incubated for another 2 h. Then 100 μ L poly HRP conjugated streptavidin substrate was incubated in each well for 20 min after washing the detection antibody, followed by incubation with 100 μ L substrate (ABTS, Thermo Scientific.) in the dark at room temperature. The absorbance was measured at 450 nm with a BioRad microplate reader. The VEGFA concentration was determined with a standard curve, and the VEGFA encapsulation efficiency (mass of VEGFA encapsulated in the microspheres / mass of VEGFA added when forming the microspheres) was calculated.

A VEGFA release test was performed in a 48-well plate at 37°C. Six mg lyophilized VEGFA-loaded Alg, Alg-g-PEG and Alg-g-RGD microspheres was dissolved in 500 μ L of PBS (pH 7.4). At each time point (1, 2, 4, 8, 12, and 24 h, followed by collection each day for a total of 14 d) 100 μ L of the PBS was removed and another 100 μ L was added to maintain the total volume. The released VEGFA concentration was determined with the DuoSet ELISA Development Kit described above.

Human MSC intracellular delivery

Green fluorescent protein (GFP)-labeled primary human MSCs were generated by lentiviral transduction used to express the gene and enriched by selecting for GFP-positive cells by fluorescence activated cell sorting (FACS) [38, 39]. DyLight 550-

labeled VEGFA (red fluorescence) was incorporated within Alg, Alg-g-PEG and Alg-g-RGD microspheres using the same above approach except the ratio of polymer to VEGFA was increased to 105:1 to increase the intensity of the visualized signal. Microspheres (500 µg/mL) were cultured with GFP-labeled primary human MSCs (100,000 cells, passage 4, green fluorescence) for 24 h in glass bottom culture dishes pre-coated with poly-d-lysine (MatTek Corporation). Confocal laser scanning (CLS) microscopy (Zeiss LSM 510 META) was used to visualize the microspheres by detecting directly through the plate. Z-stack images were formed with the use of AimImage Software.

Human MSC differentiation assays

Colorimetric osteogenesis and adipogenesis assays

Human MSCs (passage 4) were seeded in 12-well TCPS plates at a seeding density of 50,000 cells/well. Cells were cultured at 37°C and 5% CO₂ in standard MSC growth medium containing 100 µg/mL of VEGFA-encapsulated microspheres (Alg, Alg-g-PEG or Alg-g-RGD) or blank microspheres not containing VEGFA. The experiments were done in triplicate. After 48 h, old medium was aspirated off and one plate was given osteogenic differentiation medium (growth medium including 1 nM dexamethasone, 50 µg/ml ascorbic acid, 10 mMβ-glycerol phosphate) and the other plate received adipogenic differentiation medium (growth medium including 0.5 µM hydrocortisone, 0.5mM isobutylmethylxanthine, 60 µM indomethacine). Cells that served as controls were cultured without the addition of microspheres (either with VEGFA or without VEGFA). Differentiation medium was changed every 3 d. After 14 d of culture in

differentiation medium, cells were rinsed with sterile PBS and analyzed via an Alizarin Red osteogenic differentiation assay [40, 41] or an AdipoRed™ adipogenesis assay (Sigma).

In addition, six different concentrations of VEGFA ranging from 0 to 20 ng/ml (0, 0.01, 0.025, 0.05, 0.5, 20 ng/ml) were incubated with human MSCs for the control assay (extracellular VEGFA). The extracellular VEGFA differentiation experiment was conducted in 24-well TCPS plates with a seeding density of 30,000 cells/well. After 48 h, medium containing VEGFA was aspirated off and the plates were given either osteogenic or adipogenic differentiation medium. Differentiation medium was changed every 3 d. After 7 d of culture in differentiation medium, cells were rinsed with sterile PBS and analyzed via an Alizarin Red osteogenic differentiation assay [40, 41] or an AdipoRed™ adipogenic differentiation assay (Sigma).

Osteoprotegerin ELISA

Protein analysis was performed on blank (i.e., empty) and VEGFA-encapsulated Alg-g-PEG microsphere-treated hMSCs after 14 days of culture in osteogenic differentiation medium. To determine the concentration of osteoprotegerin in the supernatant of culture medium, an ELISA was performed with human osteoprotegerin ELISA DuoSet Kit according to the manufacturer's instruction.

Reverse transcription polymerase chain reaction (RT-PCR)

To quantitatively determine gene and protein information from differentiated MSCs, we selected on experimental microsphere group, Alg-g-PEG, to detect osteogenesis marker via RT-PCR and ELISA. Primary human MSCs were exposed to

VEGFA-encapsulated Alg-g-PEG microspheres, blank Alg-g-PEG microspheres or an empty control (no microspheres) for 48 h and then in osteogenic differentiation medium for 14 days. After differentiation, half of the cells were lysed to extract RNA; RNA extraction was performed using a Roche High Pure RNA Isolation Kit according to the manufacturer's instructions. Total RNA (0.25 µg) was reverse-transcribed in a 15 µl reaction using SuperScript® III CellsDirect™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The PCR reactions were conducted using 25 µl PCR super mix (Invitrogen), 2 µl of each sample and 1 µl of each primer under the following conditions: 94 °C for 5 min, followed by 35 cycles of 3.5 minutes denaturation at 94 °C, 30 s annealing at 53 °C, and 60 s extension at 72 °C. The cycles were followed by a denaturation at 72 °C for 2 min. PCR products were separated using agarose gel with 1% ethidium bromide. Primers used included: 5'-ATGAGAGCCCTCACA CTCCTC-3' (sense) and 5'-CGTAGAAGCGCCGAT AGGC-3' (antisense) for osteocalcin; 5'-GATGACACTGCCACCTCTGA-3' (sense) and 5'-GACTGGCGGGGTGTA AGT AA-3' (antisense) for core-binding factor- α 1 (RUNX2) ; 5'- GATGGATTCCAGTTC GAGTAT G-3' (sense) and 5'-GTTTGGGTTGCTTGCTCTG TTTG-3' (antisense) for collagen type I; 5'-GTCAGTGGTGGACCT GACCT-3' (sense) and 5'-AGGGGAGATTCAAGTG TGG TG-3' (antisense) for glyceraldehyde 3-phosphate dehydrogenase (GADPH) [42].

Statistical methods

All experiments were performed in triplicate; results are reported as mean \pm standard deviation. Statistical analysis was performed on the cytotoxicity data and the

differentiation data, respectively, using one-way ANOVA with Tukey multiple comparisons ($\alpha = 0.05$) via the SAS statistics program in the GLM procedure as the post-test to compare all of the groups. A $p < 0.05$ is considered significantly different.

Results

Synthesis of Alg-g-PEG and Alg-g-PEG-pyridine copolymers

The copolymer reactions for the chemical modification of alginate are shown in Fig. 1. To synthesize Alg-g-PEG-pyridine, the oligomer NH₂-PEG-S-S-pyridine was synthesized first. ¹H-NMR spectral analysis confirmed the presence of both PEG and pyridine in the final product (7.1 ppm, 7.5 ppm, 7.7 ppm, 7.9 ppm corresponding to the hydrogen on pyridine ring and 3.6 ppm corresponding to the hydrogen within the repeat group in PEG) when compared to the alginate and PEG controls (Supplemental Fig. 1). The large peak at 1.8 ppm corresponds to the excess acetate in the reaction system; this by-product does not interfere with the carbodiimide chemistry and can be removed through dialysis in the proceeding step of the copolymer reaction. Quantification of the reaction yield by peak integration determined approximately 70% reaction efficiency.

The natural polysaccharide alginate was grafted with a short chain PEG decorated with either pyridine or methyl end groups resulting in the copolymers Alg-g-PEG or Alg-g-PEG-pyridine. For the synthesis of Alg-g-PEG, ¹H-NMR spectral analysis confirmed the presence of both alginate and PEG functional groups in the final purified graft copolymers through the appearance of new peaks and peak shifts in the Alg-g-PEG spectrum (Supplemental Fig. 2); 3.36 ppm corresponds to the -CH₃ moiety of the PEG

side chain and 4.31 ppm corresponds to the shift of hydrogen atoms due to the grafting of the PEG side chain when compared to the alginate and PEG controls. As stated in the methods, the theoretical degree of PEG modification was 10 molar % of alginate backbone. Quantification of PEG conjugation by peak integration of the ^1H -NMR spectra was not successful due to extensive broadening and overlapping of peaks corresponding to alginate in the Alg-g-PEG spectra. The synthesis of Alg-g-PEG-pyridine was confirmed by ^{13}C -NMR (Supplemental Fig. 3); 132.31 ppm, 136.05 ppm, 141.37 ppm, 143.53 ppm, 149.24 ppm correspond to carbon on the pyridine ring, 69.76 ppm corresponds to the carbon within the repeat unit of PEG, and 42.84 ppm corresponds to the carbon on the alginate. Verification of NH_2 -PEG-S-S-pyridine by ^{13}C -NMR confirmed that the reaction was successful.

Fabrication of Alg, Alg-g-PEG and Alg-g-RGD microspheres

Complete microsphere fabrication strategies are shown in Fig. 2. Alg, Alg-g-PEG and Alg-g-RGD microspheres were designed to encapsulate VEGFA without interfering with the bioactivity and electrostatically-condensed structure of VEGFA. The mild gelation method by the addition of CaCl_2 during an emulsion was successful in creating microspheres encapsulating VEGFA. The surface functionalization of microspheres through CRGD conjugation did not appear to affect the shape or yield of the microspheres. SEM photomicrographs (Fig. 3A-F) demonstrate that the microspheres (Alg, Alg-g-PEG and Alg-g-RGD) were spherical in shape with an average diameters and standard deviations of approximately $1.9 \pm 1.0 \mu\text{m}$, $0.5 \pm 0.1 \mu\text{m}$, and $1.1 \pm 0.4 \mu\text{m}$, respectively (Fig. 3G). VEGFA encapsulation efficiency values were 52, 22 and 35%

respectively for the different microsphere groups; values represent an average of multiple batches within a group (n = 3).

Alg-g-PEG microspheres exhibit no cytotoxic effects

The viability of human MSCs in the presence of Alg, Alg-g-PEG and Alg-g-RGD microspheres containing no VEGFA was determined at increasing concentrations (10, 50, 100 and 500 µg/mL, Fig. 3H). The results demonstrate that Alg, Alg-g-PEG and Alg-g-RGD microspheres were non-toxic (cell viability > 90%) at concentrations up to 100 µg/mL. and cell viability was greater than 75% at a concentration as high as 500 µg/mL. A Tukey statistical test was performed to compare the experimental groups to the control groups (n = 3). No significant differences were seen between experimental microsphere groups at the same concentration (p > 0.05).

VEGFA in vitro release

VEGFA release from Alg, Alg-g-PEG and Alg-g-RGD microspheres are shown in Fig. 4 as the total percent of VEGFA released, determined from cumulative VEGFA concentrations and encapsulation efficiencies. VEGFA encapsulation efficiency values were 52, 22 and 35% respectively for the different microsphere groups (Fig. 3G). After 48 h of release, the Alg, Alg-g-PEG and Alg-g-RDG microsphere groups released 573, 802, and 1509 pg of the encapsulated VEGFA. The percentages released for the microsphere groups were 17.7, 54.2 and 61.1 %, respectively (Fig. 4A). All of the microsphere groups sustained VEGFA release for 14 d (Fig. 4B). After 14 d of release, the percentages released for the microsphere groups were 43.8, 100, and 98.2 %, respectively. When comparing the 48 h release profile with the longer 14 d release

profile, it is evident that a burst release within the first 12 h was followed by a gradual sustained release until the termination of the study.

Microsphere internalization

Internalization of Alg, Alg-g-PEG and Alg-g-RGD microspheres containing DyLight 550 (red fluorescent) labeled VEGFA was verified after examination of CLS micrographs. GFP human MSCs (green) successfully internalized DyLight-labeled VEGFA (red) encapsulated Alg, Alg-g-PEG and Alg-g-RGD microspheres (Fig. 5). The red microspheres were spread throughout the cytoplasm and appeared to surround the nucleus. The intensity of the red signal is a qualitative visualization of microsphere internalization rather than a quantitative measurement.

Human MSC differentiation

The UV absorbance of Alizarin red and fluorescent AdipoRed™ assays were used to quantify the osteogenic and adipogenic differentiation of human MSCs, respectively. The extent of differentiation was normalized to cell number by measuring the fluorescence intensity of Hoechst nuclear staining. As shown in Fig. 6A, incubation of human MSCs with microspheres containing VEGFA resulted in a significant increase in osteogenic differentiation when compared with non-modified TCPS controls. Statistical analysis of the data indicated a significant difference ($p < 0.04$) between the experimental groups (Alg, Alg-g-PEG and Alg-g-RGD) and the control group (cells cultured without the addition of VEGFA-encapsulated microspheres). The p-values for Alg, Alg-g-PEG and Alg-g-RGD microsphere groups compared to the control group were 0.0001, 0.0005 and 0.0330, respectively. For the adipogenesis differentiation assay, the Alg microsphere

group showed a significant enhancement compared to the control group ($p = 0.02$). For the other two groups (Alg-g-PEG and Alg-g-RGD), there were no significant differences for the adipogenesis assay with p-value of 0.28 and 0.57 respectively (Fig. 6B). The empty microspheres and pure VEGFA experimental groups did not exhibit any trends or significant differences for either adipogenic or osteogenic differentiation ($p > 0.06$) as shown in Fig. 6C-F. The significant differences between the experimental and control groups demonstrate that the VEGFA-encapsulated microspheres (Alg, Alg-g-PEG, Alg-g-RGD) were internalized by hMSCs and that VEGFA was delivered intracellularly, resulting in a functional output (osteogenic differentiation).

The concentration of osteoprotegerin produced by differentiated MSCs was quantified in each of the following groups: no microspheres (empty control), blank (i.e., empty) Alg-g-PEG microspheres, and VEGFA-encapsulated Alg-g-PEG microspheres (Fig. 7A). Osteoprotegerin concentrations after 14 d of culture in osteogenic medium were 205, 189, and 780 pg/mL, respectively, for the control and experimental groups. Further analysis of these results indicate a significant increase in osteogenic differentiation of MSCs after incubation with VEGFA-encapsulated Alg-g-PEG microspheres for 48 h ($p < 0.0001$).

RT-PCR analysis 14 days after culture indicated osteogenic differentiation in all the 3 treatment groups. Cells in the VEGFA-encapsulated microsphere treatment group showed greater message for osteocalcin and type I collagen, while all the treatment groups contained message for RUNX2 confirming their osteogenic identity. GAPDH was used as a loading control.

Discussion

There is a growing demand for injectable acellular therapeutics for the enhanced mineralization of osteoporotic bone. This study provides the first evidence that Alg, Alg-g-PEG and Alg-g-RGD microspheres, fabricated from graft copolymers of alginate and amine-terminated PEG or alginate and pyridine-conjugated PEG, may be used for the controlled intracellular delivery of VEGFA into living human stem/progenitor cells for enhanced osteogenic differentiation. Through the use of a biodegradable natural polymer, mild gelation methods, chemical conjugation of PEG, and immobilization of the adhesion ligand RGD, the applicability of controlled drug delivery by means of microspheres increases. The Alg, Alg-g-PEG and Alg-g-RGD microspheres were uniform in shape, of a moderate size to allow for internalization into human MSCs, and proved adaptable to surface-modification [34, 35]. The fabrication was easy to perform and translate to engineering applications compared to other approaches which require higher spinning speeds and double emulsion processes.

VEGFA release from alginate-based microspheres was sustained over a period of 14 d, which is promising for targeted intracellular delivery. However, the intracellular delivery of VEGFA into human MSCs took place over a shorter time-span of 48 h. The non-selectivity of the microspheres in culture with MSCs allowed uptake to occur quickly, and this is also believed to be the case for the internalization of microspheres during the differentiation assay; the microsphere concentration and duration of co-culture were consistent among the internalization and differentiation studies. The short time frame allowed for delivery also suggests that the VEGFA content of the internalized

microspheres was high enough to significantly affect MSC differentiation. Drug diffusion may be hindered by PEGylation of the alginate; an effect that has been reported for Alg-g-PEG chains of moderate to high molecular weights [19]. The varying release kinetics with time suggest that the microsphere structure may be optimized further for controlled release applications [43]. Decreasing the variability in the diameter of the microspheres for all three experimental groups may result in different encapsulation efficiencies and/or release rates. However, microsphere size homogeneity and drug release profile optimization were outside the scope of this study and may be addressed through further investigations. Indeed, in regards to the MSC differentiation experiments, encapsulation efficiency was a more critical parameter compared to the in vitro extracellular release kinetics of the microspheres.

Multiple uptake pathways have been targeted as a means of delivering material intracellularly, namely endocytosis, in the case of cationic nanoparticles [44] or for larger micron-scale particles [45]. Due to the mild and quick gelation used to fabricate Alg, Alg-g-PEG and Alg-g-RGD microspheres, encapsulated drugs and proteins are better protected. Chemical conjugation of RGD onto PEG utilized disulfide bonds, which are covalent linkages arising from the oxidation of two sulfhydryl (SH) groups of cysteine connected to RGD (CRGD) and the SH terminal group on PEG (SH-PEG-NH₂). Disulfide bonds exist commonly in eukaryotic cell proteins with the function of fortifying the protein tertiary structure. Due to the reversibility and relative stability in plasma, the disulfide bond linkage becomes attractive in designing drug delivery systems. In addition, the physical properties and network structure of the calcium crosslinked alginate-based microspheres are readily changed after alterations in pH (such as in an endosome) or in

the presence of calcium chelators. For the system described herein, it is hypothesized that the microspheres rapidly released VEGFA into the cytoplasm once inside the MSCs [46, 47] due to the disassembly of the microspheres.

RGD is widely utilized in tissue engineering studies and has been used in studies with alginate [48, 49]. In the current study RGD was used a model ligand which may be replaced with more relevant cell-recognition molecules for targeting MSCs *in vivo*.

However, RGD has been shown to increase uptake of surface modified DNA complexes for intracellular delivery [50]. Although a limitation of the current study was in the collection of qualitative internalization results, future work will investigate RGD modified microspheres for enhanced MSC uptake and it may be combined with other cell-recognition motifs to control drug delivery for target cell populations.

Results of the differentiation colorimetric assays did not exhibit significant enhancement of the RGD or PEG modification between alginate microsphere groups in promoting osteoblast differentiation. Alg-g-PEG microspheres were chosen to further quantify the significance of the intracellular delivery method of VEGFA-encapsulated microspheres. MSCs in the VEGFA-encapsulated microsphere treatment group showed greater message for osteocalcin and type I collagen compared to the control groups. These results are promising and warrant further investigation into the intracrine activation mechanisms of growth factors for regulating MSC differentiation.

Although Alg-g-RGD microspheres resulted in the lowest Alizarin red absorbance, several reasons may account for this. The cells in the current study were only directed to differentiate for 14 d, meaning that the human MSCs cultured with Alg-g-RGD microspheres might still result in enhanced osteogenesis but more time is needed to

demonstrate that result. Another possible explanation is RGD's influence on differentiation signaling. The research of Garcia et al. (2005) and Park et al. (2010) indicate a promotion effect of RGD in stem cell differentiation [33, 51]. In addition, it could also enhance chondrogenic differentiation [52]. However, other studies present findings in disagreement with the aforementioned work [53, 54]. The potential for Alg, Alg-g-PEG and Alg-g-RGD microspheres to deliver VEGFA intracellularly may be applied to investigate a wide array of differentiation lineages for MSCs and perhaps other progenitor cells.

Of special interest, our functional tests with VEGFA-bearing microspheres support an intracrine mechanism for VEGFA in human MSCs and are consistent with results reported by Liu et al. (2012) with retroviral expression of VEGFA in murine MSCs. We found that intracellular delivery of VEGFA via alginate-based microspheres resulted in up-regulated osteogenic differentiation by human MSCs, suggesting the effectiveness of our drug delivery system to control the intracrine mechanism that balances osteoblast and adipocyte activity. Extracellular VEGFA did not alter differentiation of human MSCs. To carefully evaluate the function of intracellular VEGFA in regard to specificity for bone formation, we used adipogenesis as an alternative differentiation out-put. MSCs from osteoporotic bones exhibit a higher adipogenic capacity compared to healthy MSC donors, which is why adipogenic differentiation was chosen as the alternative lineage [55]. Except for the Alg microsphere group, in which adipogenesis by MSCs was enhanced, there was not a significant difference in adipogenesis for MSCs incubated with the VEGFA-encapsulated Alg-g-PEG and Alg-g-RGD microspheres. Based on the enhanced adipogenesis we observed in

the VEGFA-encapsulated Alg microspheres group, Alg-g-PEG and Alg-g-RGD microspheres may be better suited for controlled intracellular delivery to enhance osteogenesis *in vivo*. The investigation of various cell-recognition molecules (i.e. ligands, antibodies, etc.) may provide a viable option for targeting a specific cell population *in vivo* for enhanced mineralization of osteoporotic bone.

Conclusion

This study is the first to report the fabrication and surface-functionalization of Alg, Alg-g-PEG and Alg-g-RGD microspheres for the encapsulation and intracellular delivery of a bioactive growth factor, VEGFA. Alg, Alg-g-PEG and Alg-g-RGD microspheres containing VEGFA promoted osteogenic differentiation of human MSCs upon intracellular delivery after 48 h of incubation time. These significant results provide encouraging evidence for development of a systemic growth factor delivery system with potential to treat many debilitating diseases. Future work will involve manipulation of the surface ligand to enhance cell-targeted internalization of microspheres for intracellular growth factor delivery.

Acknowledgements

The authors gratefully acknowledge members of the Engineered Biomaterials Research Laboratory at the University of Vermont, the visiting NSF-REU research assistant Ashleigh Bristol from University of Mississippi for technical assistance, Alexander Aronshtam, Ph.D. for cloning and purification of human VEGFA, and Jianjin Wang for assistance with the statistical analysis. The authors would also like to thank members of the University of Vermont Microscopy Imaging Center in the College of Medicine for

assistance with CLS and SEM imaging. This work was supported by the College of Engineering and Mathematical Sciences at the University of Vermont and NIH R01 HL085210 (to J.L.S.).

SUPPORTING INFORMATION AVAILABLE

NH₂-S-S-pyridine was verified via ¹H-NMR spectra (500 MHz, D₂O) as shown in Supplementary Fig. 1. The peaks within the range of 7-8 ppm confirmed the success of the chemistry. ¹H-NMR analysis of Alg-g-PEG was performed to verify the retention of PEG after the grafting reaction. Alg-g-PEG was synthesized using mPEG-NH₂ and sodium alginate via carbodiimide chemistry (Supplementary Fig. 2). ¹H-NMR spectra (500 MHz, D₂O) are shown, comparing the graft copolymer Alg-g-PEG to the homopolymer constituents: mPEG-NH₂ and sodium alginate. The inset image is the chemical structure of the Alg-g-PEG copolymer. The alginate conjugation onto NH₂-S-S-pyridine which forms the Alg-g-pyridine was confirmed by ¹³C-NMR (500 MHz, D₂O) (Supplementary Fig. 3).

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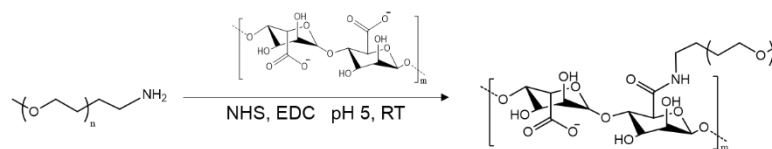
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A Synthesis of Alg-g-PEG



B Synthesis of Alg-g-PEG-S-S-pyridine

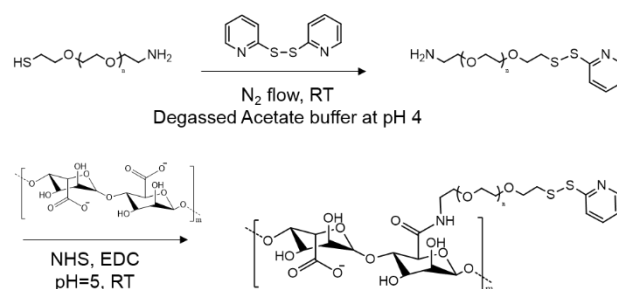


Figure A1- 1. Chemical modification of alginate (Alg) with two different poly(ethylene glycol) (PEG) oligomers with methyl and pyridine end groups, respectively

The synthesis of Alg-g-PEG was conducted using carbodiimide chemistry at pH 5 and room temperature. To synthesize Alg-g-PEG-S-S-Pyridine, NH₂-PEG-SH was reacted with 2,2'-dithiodipyridine in degassed acetate buffer at pH 4 and room temperature under N₂ flow. Next, the modified NH₂-PEG-pyridine was conjugated to alginate using carbodiimide chemistry.

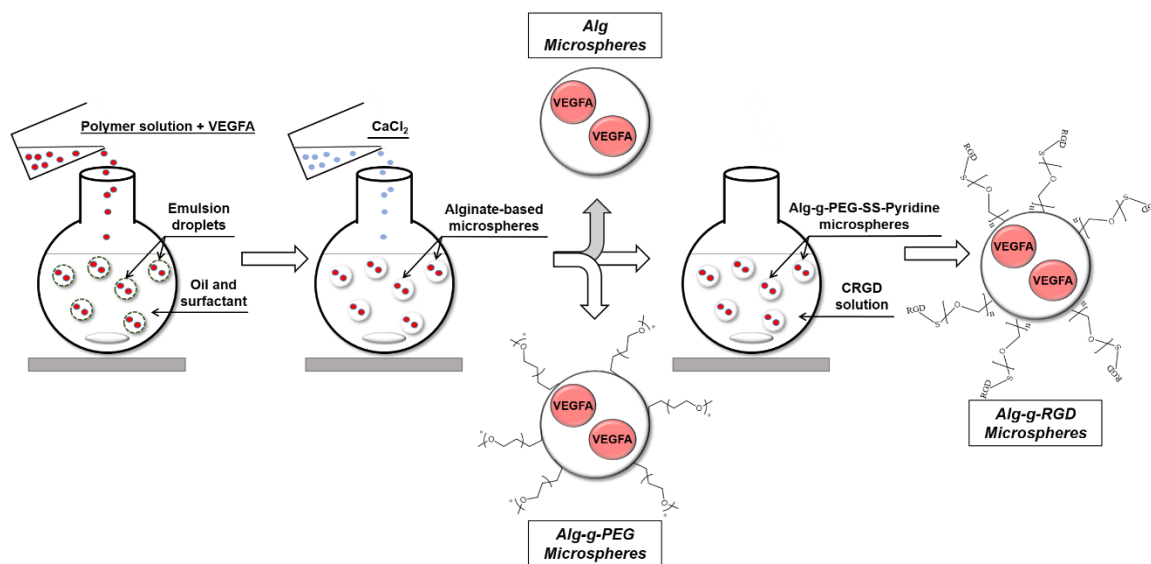


Figure A1- 2. Schematic representation of microsphere fabrication techniques

Microspheres with or without VEGFA were prepared by premixing alginate or alginate-based copolymer solutions with VEGFA and creating a water/oil emulsion at room temperature in the presence of 1M calcium chloride. An additional surface modification step was performed on Alg-g-PEG-S-S-Pyridine microspheres to chemically conjugate CRGD via disulfide bonds. For Alg-g-RGD microspheres, the conjugation of 2,2'-dithiodipyridine was used to exchange the thiol group on the cysteine-RGD (CRGD) after microsphere fabrication.

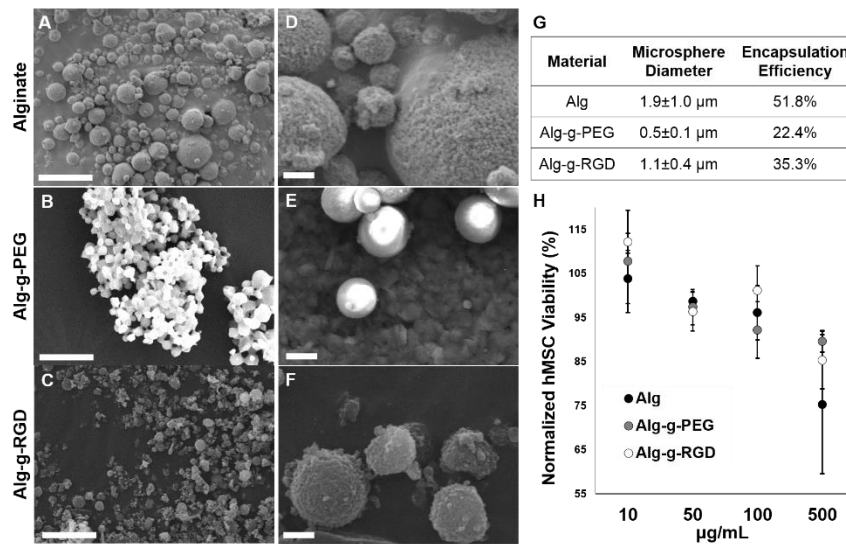


Figure A1- 3. The shape, average diameter, VEGFA encapsulation efficiency and cytotoxicity of alginate-based microspheres

These were verified using scanning electron microscopy (SEM), an ELISA assay ($n = 3$), and an MTT-based In Vitro Toxicology assay. SEM micrographs representing (A,D) Alg, (B,E) Alg-g-PEG, and (C,F) Alg-g-RGD microspheres were fabricated without the addition of VEGFA through a water/oil emulsion; micrographs shown in A,B,C have a scale bar = 10 µm while micrographs in D,E,F have a scale bar = 1 µm. In preparation for imaging, microspheres were frozen by immersion in liquid N₂ and subsequently lyophilized; samples were sputter-coated with 45 nm of Au-Pb. (G) Microsphere diameters were determined using ImageJ analysis on SEM micrographs. (H) The effect of microsphere concentration (10, 50, 100, and 500 µg/mL) on primary human MSC viability after 24 h culture in standard MSC growth medium at 37°C and 5% CO₂ was determined. Experimental groups were normalized to non-modified confluent human MSCs cultured on tissue culture polystyrene. A Tukey statistical test was performed. No significant differences were seen between groups at the same concentration.

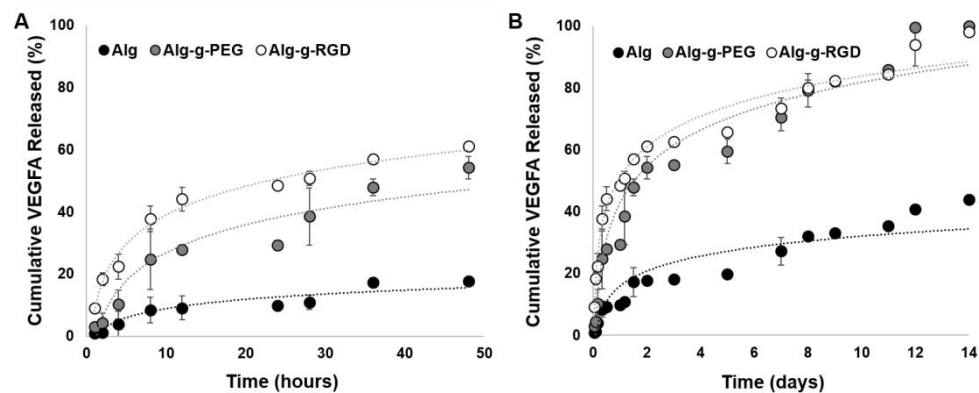


Figure A1- 4. Quantitative release of VEGFA (%) was calculated using the encapsulation efficiencies of each sample group and the VEGFA concentration after each time point

Sample aliquots were collected and VEGFA concentration was determined using an ELISA assay (n = 3). (A) Cumulative VEGFA release (ng/mL) from alginate-based microspheres in PBS at pH 7.4 and 37°C over a sampling period of 48 h and (B) 14 d was determined.

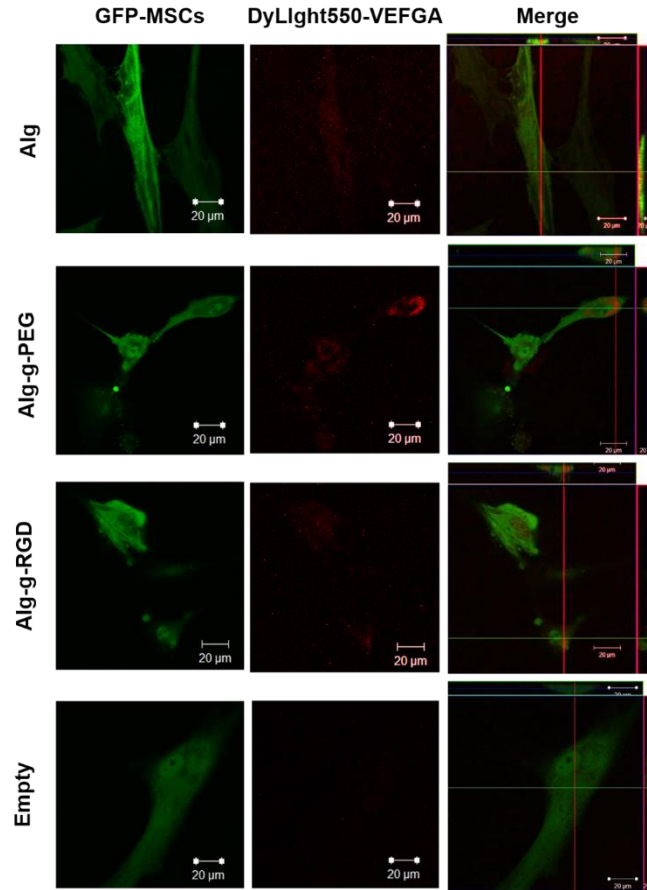


Figure A1- 5. Confocal light microscopy images

GFP-labeled human MSCs (green) after 24 h of culture with DyLight 550 labeled-VEGFA-encapsulated alginate-based microspheres (red) at a concentration of 500 $\mu\text{g/mL}$ in standard MSC growth medium. The Alg, Alg-g-PEG and Alg-g-RGD images all verify the internalization of microspheres, especially compared to the non-modified empty Alg microsphere control. The red intensity is a qualitative visualization of microspheres internalization rather than a quantitative measurement.

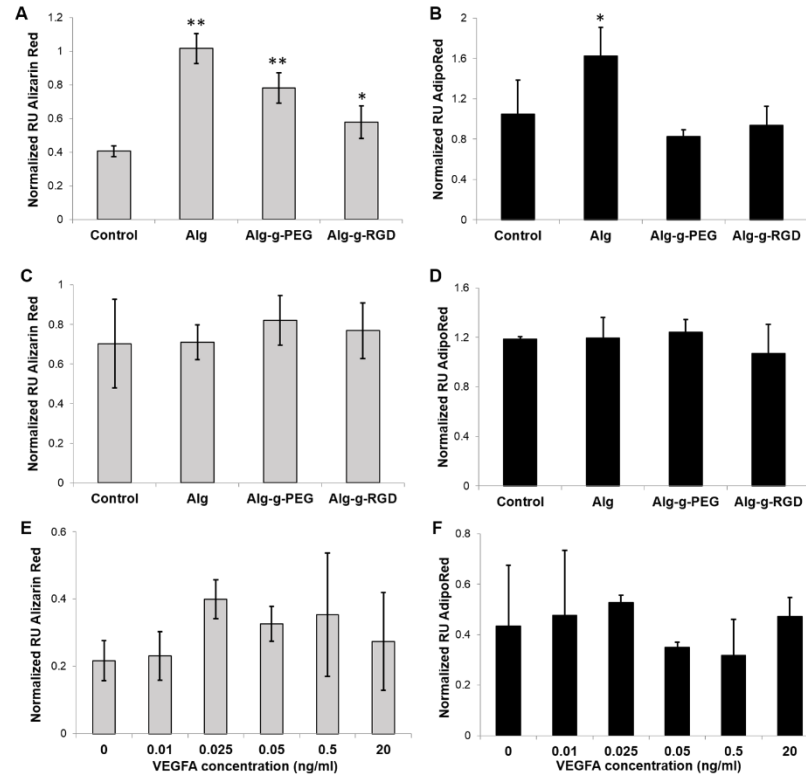


Figure A1- 6. In vitro osteogenic (gray) and adipogenic (black) differentiation assay results of human MSCs after 14 days in culture with differentiation growth medium

(A) and (B) represent the VEGFA-encapsulated microspheres, (C) and (D) represent empty microspheres, and (E) and (F) represent extracellular delivery of pure VEGFA at different concentrations ranging from 0 to 20 ng/ml. A Tukey statistical test was performed to compare the experimental groups to the control groups ($n = 3$). ** shows a significant p-value less than 0.0005 and * shows p-value less than 0.04. For the control group, only standard MSC growth medium was added. The graphs illustrate a significant difference between VEGFA encapsulated Alg, Alg-g-PEG and Alg-g-RGD microsphere groups compared to the control group in the osteogenesis assay as well as for the VEGFA encapsulated Alg microsphere group in the adipogenesis group. No significant differences were seen ($p \leq 0.05$) between control and experimental groups for the osteogenesis and adipogenesis assays when empty microspheres were used or VEGFA was delivered extracellularly.

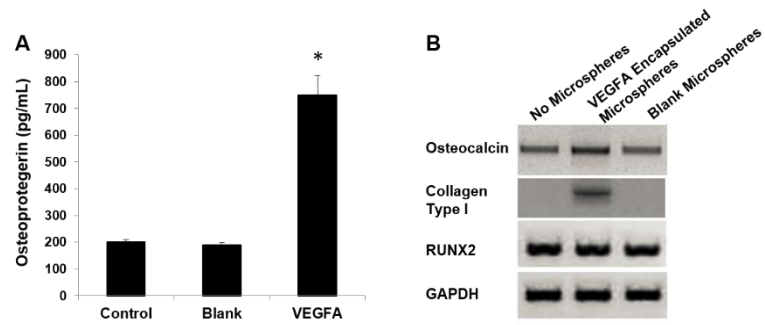


Figure A1- 7. In vitro osteogenic differentiation results

(A) Protein results of human MSCs after 14 days in culture with differentiation growth medium. Sample aliquots were collected and osteoprotegerin concentration (pg/mL) in the supernatant medium was measured using an ELISA assay (n = 3). * indicates significantly higher result, $p < 0.0001$. (B) RT-PCR results verify production of osteocalcin and RUNX2 in MSCs after 14 days in culture with osteogenic differentiation growth medium. Collagen type I was upregulated for MSCs pre-treated with VEGFA-encapsulated Alg-g-PEG microspheres prior to differentiation. GAPDH was used as an internal control.

APPENDIX II. PRECLINICAL TESTING AND INTELLECTUAL PROPERTY ASSOCIATED WITH THIS PHD DISSERTATION

Preclinical tests of therapeutics from Spees Lab

(A) Intracoronary infusion of EPI CdM or HGF/IgG complexes in adult swine with MI. To ascertain whether EPDC CM could preserve cardiac tissue after MI in a large animal model, our group has performed a short-term preliminary treatment study with 10 adult commercial swine (~65 kgs). Under anesthesia and with fluoroscopic guidance, a balloon catheter was advanced and inflated to completely occlude the LAD for 60 min prior to revascularization. At the time of reperfusion, pigs in the control group received additional contrast dye to confirm re-flow (n=5). Alternatively, pigs in the treatment groups received 16 mls of 20x EPDC CM and contrast (n=3) or 16 mls of 25x EPDC CM and contrast (n=2), by infusing EPDC CM directly into the LAD at the location of the deflated balloon. The EPDC CM was infused over 3-5 min. No anti-arrhythmic drugs were used and there were no adverse events following any of the treatments. All pigs were euthanized at 24 hr after treatment. Hearts were removed and cut transversely from apex to base (1 cm slices), stained by TTC, and digitally photographed (Figure 1A). The weights of right and left ventricular tissue from each 1 cm slice were recorded, while areas of viable and scarred cardiac tissue were quantified (Scion Image 4.0.2) and multiplied by the tissue weights to estimate infarct size. As the effects of 20x and 25x CM on infarct size were similar, treatment data were together ($p=0.024$, $n=5$; Figure 1B).

Using the same PCI-based ischemia/reperfusion MI model, a pilot study to test an effective intracoronary dose of HGF/IgG complexes has also been performed in adult

swine. From the guide catheter, at the time of reperfusion, either alpha MEM (vehicle, n=2) or HGF/IgG complexes (0.125 mg/50 kg pig, n=2) was infused, followed by similar method for tissue processing analysis as before. Initial observations by comparing Evan's Blue staining and TTC areas at risk (red; viable) are presented here, in animals treated with HGF/IgG complexes (Figure 1C, C').

(B) Subepicardial engraftment of primary human EPicardial Derived Cells (EPDCs) into adult swine with MI.

Preliminary results indicated that human EPDCs could be primed in CTGF-D4/Ins to for engraftment into immunocompromised pigs after MI, at the time of reperfusion. Human cells were detected at 1 week following engraftment by staining for human antigen Alu, on coronal sections of the pig heart (Figure 1D).

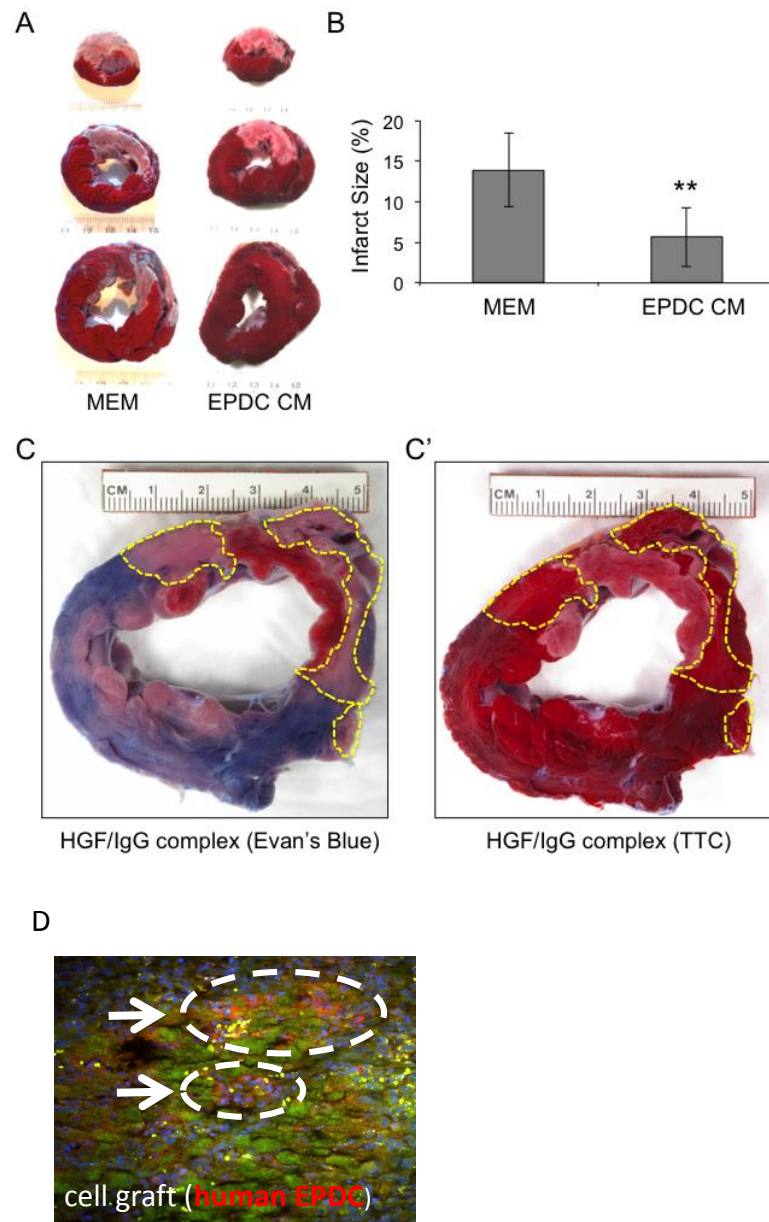


Figure A2- 1. Adult swine model of MI

A) Representative 1 cm slices from control (MEM) and CM-treated hearts stained by TTC at 24 hr after 60 min of ischemia with reperfusion. Note that the non-viable (white) area in the control heart extends through the 3rd section in both the LV and RV (far left section), whereas the infarct in the CM-treated heart does not. B) EPDC CM treatment significantly reduces infarct size 24 hr after MI ($p=0.024$, MEM vs. CM-treated, $n=5$ each). Error bars, SD of mean. C, C') HGF/IgG treatment promotes myocardial rescue within the Evan's Blue risk region. Note area within the yellow dashes stained red (viable) by TTC in (C'), but is within the risk region for low/no perfusion after MI (non-blue area delineated by yellow dashes in C). (D) Representative tissue section of adult swine heart 1 week after MI, grafted with human EPDC at the time of reperfusion.

Intellectual Property

1. Compositions and methods for cardiac tissue repair

USPN 9,132,155 [Issued](#): Sept 15, 2015

U.S. Patent Application No. 14/826,613, filed on August 14, 2015

The invention features compositions comprising epicardial progenitor cells and agents having cardiac protective activity isolated from epicardial progenitor cells and derivatives thereof, and methods for the use of such compositions.

2. Compositions and Methods for Cardiac Tissue Repair (Cell-Kro)

U.S. Patent Application No. 14/771,747, filed on Aug 31, 2015 (Mar 8, 2012)

EU Patent Application No. 14 760 147.0, filed on Mar 8, 2013 (Mar 8, 2012)

The invention features compositions featuring (a) one or more of connective tissue growth factor (CTGF) and human C-terminal CTGF peptide; and (b) one or more of insulin and IGF-1; and methods of using such compositions to reduce cardiac tissue damage associated with an ischemic event or to enhance engraftment of a cell in a cardiac tissue.

3. Compositions and Methods for Vascular Protection against Reperfusion Injury after Myocardial Ischemia

U.S. Patent Application No. 62/126,374, filed on Feb 27, 2015

The invention features compositions featuring (a) one or more of human hepatocyte growth factor (HGF) and (b) one or more of Immunoglobulin G (IgG); and methods of using such compositions to protect vascular cells against reperfusion injury in order to reduce cardiac tissue damage associated with an ischemic event.